5-2018

# Protein Trafficking of BK Channel β1 Subunits in Cerebral Artery Myocytes

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## Abstract

Rationale: Large-conductance calcium ($Ca^{2+}$)-activated potassium channels (BK) are expressed in arterial myocytes to control arterial contractility. It is composed of pore- forming BKα and auxiliary β1 subunits. Auxiliary β1 subunits associate with BKα which modulate $Ca^{2+}$ sensitivity of BK channel. Previous data showed that BKα locates at cell membrane, whereas β1 subunits are primarily intracellular which regulated by Rab11A- positive recycling endosomes. Endothelin-1 (ET-1), a vasoconstrictor, induces contraction of myocytes. ET-1 inhibits BK channel but mechanisms are not fully understood. It is unclear that vasoconstrictors regulate the cellular distribution of BK channels. Furthermore, BK channels are involved in hypertension. Hypertension increases risk of major cardiovascular and cerebrovascular events, such as stroke and mental dysfunction. During hypertension, cerebral arteries have high myogenic tone and are less responsive to vasodilators, including nitric oxide (NO). The regulation of arterial contractility by BK channels is altered during hypertension, although mechanisms involved are also unclear.

Objective: Test the hypothesis that ET-1 inhibits β1 surface trafficking in myocytes via activation of PKC. Test the hypothesis that activation of PKC directly modulates Rab11A through phosphorylation. Furthermore, test the hypothesis that trafficking of pore- forming BK channel (BKα) and auxiliary β1 subunits contributes to pathological changes in contractility in cerebral arteries of stroke-prone spontaneously hypertensive rats (SP- SHRs).

Methods and Results: ET-1 decreased NO-induced or depolarization-induced surface β1 expression and association with BKα in myocytes through activation of protein kinase C (PKC). Total β1, total BKα proteins or surface BKα was not altered by ET-1. Rab11A regulates β1 protein trafficking in Rab11A-positive recycling endosome. ET-1 reduced Rab11 activity via phosphorylation. Five probable phosphorylated sites on Rab11A were identified, among which Ser177 has highest probability. A phosphorylation-mute Rab11A construct (Rab11A S177A) or wild-type Rab11A construct similarly increased total Rab11A protein in transfected myocytes. Rab11A S177A inhibited ET-1-reduced Rab11A activity and decreased β1 protein trafficking. Rab11A S177A reversed PKC- dependent block of single BK channels and transient BK currents in myocytes. Rab11A S177A partially blocked ET-1-induced vasoconstriction. In contrast, NO-induced surface-trafficking of β1 subunits, BK current activity and vasodilation did not involve Rab11A S177. Our data also indicate that the amounts of total and surface BKα and β1 subunits were similar in unstimulated arteries of SP-SHRs and age-matched, normotensive Wistar-Kyoto rat controls. In contrast, the stimulated surface-trafficking of β1 subunits by either NO (sodium nitroprusside, SNP) or membrane depolarization was inhibited in SP-SHR arteries. BIM, a PKC inhibitor, and overexpression of a mutant Rab11A construct that cannot be phosphorylated by PKC at serine 177 (Rab11A S177A) restored the stimulated surface-trafficking of β1 subunits. PKC-mediated inhibition of β1 trafficking prevented BK channel activation by NO in arterial myocytes of SP-SHRs and this was restored by the expression of Rab11A S177A, but not by Rab11A. Vasodilation to NO and lithocholate, an activator of β1 subunit-containing BK channels, was inhibited in pressurized arteries of SP-SHRs. Vasodilation to these agents was reestablished by BIM in SP-SHR arteries.

Conclusions: In smooth muscle cell, ET-1 activates protein kinase C which phosphorylates Rab11A at Ser177 to reduce Rab11A activity. Inhibition of Rab11A blocks anterograde trafficking of β1 subunits to associate with BKα on cell surface. Less β1 subunits reduces $Ca^{2+}$ sensitivity of BK channel and transient BK channel currents which leads to vasoconstriction. Spontaneously active PKC inhibits β1 subunit trafficking in arterial myocytes and is responsible for dysfunctional NO-induced BK channel activation and vasodilation in cerebral arteries of SP-SHRs.

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A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Xue Zhai
May 2018

## DEDICATION

I dedicate this dissertation
to my parents, Yuejin Zhai and Jin Zhang,
to my fiancée Yichen Sun,
to all my extended family,
for their gracious love and support.

## ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Jonathan H. Jaggar, for his great and kindly supervision and tremendous support of my studies. Without his mentorship and encouragement, I won't be able to fulfill my dream. He has guided me how to design the research, do experiments, make presentations, and write papers. I am very appreciate his mentorship for the graduate education to lead my future career.

I give my sincere gratitude to my dissertation committee members Drs. Adebowale Adebiyi, William Earl Armstrong, Julio Cordero-Morales and Elena Parfenova for their valuable suggestions and insightful comments during my journey to phD.

I am grateful for the lab instructor, Dr. Marie Dennis M. Leo who helped me design the project and taught me the relevant techniques. I really appreciate Dr. Qian Wang for teaching me the patch clamp technique. It was very precious time companioned by her during that difficult time. I also thank lab former and current colleagues Dr. John P. Bannister, Kirk W. Evanson, Raquib Hasan, Priya Muralidharan and Michael W. Kidd for their kind assistance for my research studies.

I would like to thank the University of Tennessee Health Science Center for give me the awesome opportunity to purse doctorate degree. My appreciation also gives the Department of Physiology for the facilities, seminars and all kinds of assistantship. I also sincerely thank Mr. and Mrs. Gerwin for awarding me the Gerwin Graduate Scholarship to support my research.

I am forever indebted to my patients and my extended family for their unconditional love, care and support. Finally, praise the Lord and give glory to God for everything I have achieved today.

## ABSTRACT

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to NO and lithocholate, an activator of β1 subunit-containing BK channels, was inhibited in pressurized arteries of SP-SHRs. Vasodilation to these agents was reestablished by BIM in SP-SHR arteries.

**Conclusions:** In smooth muscle cell, ET-1 activates protein kinase C which phosphorylates Rab11A at Ser177 to reduce Rab11A activity. Inhibition of Rab11A blocks anterograde trafficking of β1 subunits to associate with BKα on cell surface. Less β1 subunits reduces $Ca^{2+}$ sensitivity of BK channel and transient BK channel currents which leads to vasoconstriction. Spontaneously active PKC inhibits β1 subunit trafficking in arterial myocytes and is responsible for dysfunctional NO-induced BK channel activation and vasodilation in cerebral arteries of SP-SHRs.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine 5’-diphosphate |
| Ang II | Angiotensin II |
| ATPase | Adenosine 5’-triphosphatase |
| BSA | Bovine serum albumin |
| BK channel | Large conductance calcium-activated potassium channel |
| BP | Blood pressure |
| $Ca^{2+}$ | Calcium |
| $Ca_V1.2$ | L-type voltage-dependent Ca2+ channel |
| $Cl^-$ | Chloride |
| CO | Cardiac output |
| CO | Carbon monoxide |
| DAG | Diacylglycerol |
| DMEM | Dulbecco's modified eagle medium |
| eNOS | Endothelial NOS |
| ERK | Extracellular signal-regulated kinases |
| ER | Endoplasmic reticulum |
| ET-1 | Endothelin-1 |
| FBS | Fetal bovine serum |
| $H^+$ | Hydrogen |
| $H_2O_2$ | Hydrogen peroxide |
| HEK293 cells | Human embryonic kidney 293 cells |
| $IP_3$ | Inositol 1,4,5-triphosphate |
| $K^+$ | Potassium |
| kDa | Kilodalton |
| $Mg^{2+}$ | Magnesium |
| MAPK | Mitogen-activated protein kinases |
| MLC | Myosin light chains |
| MLCK | Myosin light chain kinase |
| $Na^+$ | Sodium |
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NO | Nitric oxide |
| NOS | NO synthase |
| $O^2$ | Molecular oxygen |
| $O^{2-\cdot}$ | Superoxide |
| $OH\cdot$ | Hydroxyl radical |
| PBS | Phosphate-buffered saline |
| PI3K | Phosphatidylinositol 3-kinase |
| PIP2 | Phosphatidylinositol 4,5-biphosphate |
| $PIP_3$ | Phosphatidylinositol-3 |
| PK | Protein kinase |
| PKA | Protein kinase A |
| PKB | Protein kinase B |

| | |
|---|---|
| PKC | Protein kinase C |
| PKG | Protein kinase G |
| PLC | Phospholipase C |
| PSS | Physiological salt solution |
| ROS | Reactive oxygen species |
| RyR | Ryanodine-sensitive $Ca^{2+}$ channel |
| siRNA | Small interfering RNA |
| SR | Sarcoplasmic reticulum |
| STOC | Spontaneous transient outward current |
| SVR | Systemic vascular resistance |
| TBS | Tris-buffered solution |
| TBS-T | TBS with 0.1% tween-20 |
| VDCC | Voltage-dependent $Ca^{2+}$ channel |

# CHAPTER 1. INTRODUCTION

## Cardiovascular System

Cardiovascular system consists of vascular tubes that transports blood from heart to peripheral tissues and then returns blood back to heart. Vascular system contains various types of blood vessels in human body, i.e., arteries, arterioles, capillaries, venues and veins. (Eric Widmaier 2002) Three layers have been identified within blood vessels, that is, tunica intima, tunica media and tunica adventitia. (Eric Widmaier 2002) Tunica intima is a simple squamous epithelium which is called endothelium. (Eric Widmaier 2002) The inner part of entire cardiovascular system is covered by the continuous layer of endothelium. (Eric Widmaier 2002) Tunica media locates in the middle of vascular wall which contains smooth muscle cells. (Eric Widmaier 2002) Tunica adventitia is made of connective tissue as well as nerves that go with it. (Eric Widmaier 2002) Arterial vessels transport oxygenized blood to tissues to provide oxygen and nutrition. (Eric Widmaier 2002) Venous vessels drain the deoxygenized blood back to heart. (Eric Widmaier 2002) Deoxygenized blood goes through right atrial to right ventricle where it is pumped into pulmonary arteries. (Eric Widmaier 2002) In the lung, deoxygenized blood exchanges its carbon dioxide with oxygen and turns into oxygenized blood. (Eric Widmaier 2002)

Blood vessel size is regulated by changing its diameter of lumen. Constriction or dilation of arteries is controlled by contraction or relaxation of vascular smooth muscle cells, respectively. Sympathetic fibers of autonomic nervous system innervate vascular smooth muscle. (Eric Widmaier 2002) An increase in sympathetic output stimulates myocytes to contract, resulting in narrowing the lumen. A decrease in the diameter of the lumen of a blood vessel is called vasoconstriction. In contrast, vasodilation is the process of blood vessel dilation leading to an increase in lumen diameter. (Eric Widmaier 2002)

Vascular resistance is the product of vessel length and blood viscosity, reversed by radius. (Eric Widmaier 2002) The blood pressure is the product of cardiac output (CO) and systemic vascular resistance (SVR). (Eric Widmaier 2002) The equation is $BP = CO \times SVR$. So increased intravascular volume or increased systemic vascular resistance results in elevated blood pressure.

## Vascular Smooth Muscle Cells

Smooth muscle cells, or myocytes are located in tunica media of blood vessels. (Eric Widmaier 2002) Myocytes are innervated by sympathetic nerves through adrenoceptors such as $\alpha 1$, $\alpha 2$, $\beta 2$ receptor. (Eric Widmaier 2002) Like skeletal muscle, myocytes contain thick myosin filaments and thin actin filaments. (Eric Widmaier 2002) Moreover, intermediate filaments attach to the structures called dense bodies in smooth muscle cells. (Eric Widmaier 2002)

## $Ca^{2+}$ dependent smooth muscle contraction

Intracellular $Ca^{2+}$ concentration controls the contractile activity in myocytes. (Brozovich, Nicholson et al. 2016) $Ca^{2+}$ stimulates cross-bridge cycle between myosin and actin which is controlled by $Ca^{2+}$ regulated enzyme. (**Figure 1-1**) Intracellular $Ca^{2+}$ concentration maintains a basal level of cross-bridge activity which is known as smooth muscle tone. (Brozovich, Nicholson et al. 2016) Intracellular $Ca^{2+}$ binds to calmodulin. $Ca^{2+}$ calmodulin complex binds to a 20 kDa regulatory Ser/Thr kinase called myosin light chain kinase (MLCK) therefore $Ca^{2+}$ calmodulin complex activates MLCK. MLCK is a $Ca^{2+}$/ Calmodulin dependent kinase which is stimulated by intracellular $Ca^{2+}$ rise. Active MLCK uses ATP to phosphorylate myosin light chains. (**Figure 1-1**) Smooth muscle myosin is phosphorylated at Ser 19 of myosin light chain kinase (MLCK). (Ito and Hartshorne 1990) Then phosphorylated myosin moves the cross-bridge to bind to actin which results in contraction of smooth muscle cell. (Ito and Hartshorne 1990) Dephosphorylation occurs during relaxation of myocytes because dephosphorylated myosin did not bind to actin. Myosin phosphatase decreases myosin activity by dephosphorylation of myosin. (Archer, Huang et al.)

## $Ca^{2+}$ independent smooth muscle contraction

The process of $Ca^{2+}$ independent contraction mechanism is dependent on MLCK activation with subsequent actin-myosin cross-bridging. (Brozovich, Nicholson et al. 2016) Myosin light chain phosphatase (MLCP) removes the high-energy phosphate group from myosin thereby causing relaxation. (Brozovich, Nicholson et al. 2016) RhoA/Rho kinase pathway inhibits dephosphorylation of myosin light chain by myosin light chain phosphatase. (Schmidt and Hall 2002) RhoA is activated by exchanging of GDP for GTP. Active RhoA stimulates Rho kinase, a serine/threonine kinase. Rho kinase inhibits MLCP by phosphorylation, thus prevents myosin light chain dephosphorylation in order to maintain contraction. (Brozovich, Nicholson et al. 2016) Furthermore, actin binding protein caldesmon is phosphorylated by ERK. (Bryan 1990, Wang, Wang et al. 1991) Caldesmon in myocytes functions as troponin complex in striated muscle which it inhibits the ATPase activity of myosin and blocks the cross-bridge of myosin and action. (Bryan 1990, Wang, Wang et al. 1991) Myosin ATPase activity is inhibited by C terminus of caldesmon. (Bryan 1990, Wang, Wang et al. 1991)

## BK Channel

Large-conductance $Ca^{2+}$-activated potassium (BK) channel, also known as Slo1/MaxiK/KCa1.1 channel, expresses in a various types of tissues. (Jaggar, Porter et al. 2000) BK channel most commonly expresses in neuron as well as smooth muscle cells. It is also detected in liver, spleen, pancreas, adrenal glands, ovary and testes. As its term indicates, BK channel exhibits a large conductance (~ 250-300 pS) in symmetrical 150 mM $K^+$. (Jaggar, Porter et al. 2000) BK channel functions in maintaining the dynamic regulation between vasoconstriction and vasodilation. Activation of BK channels results

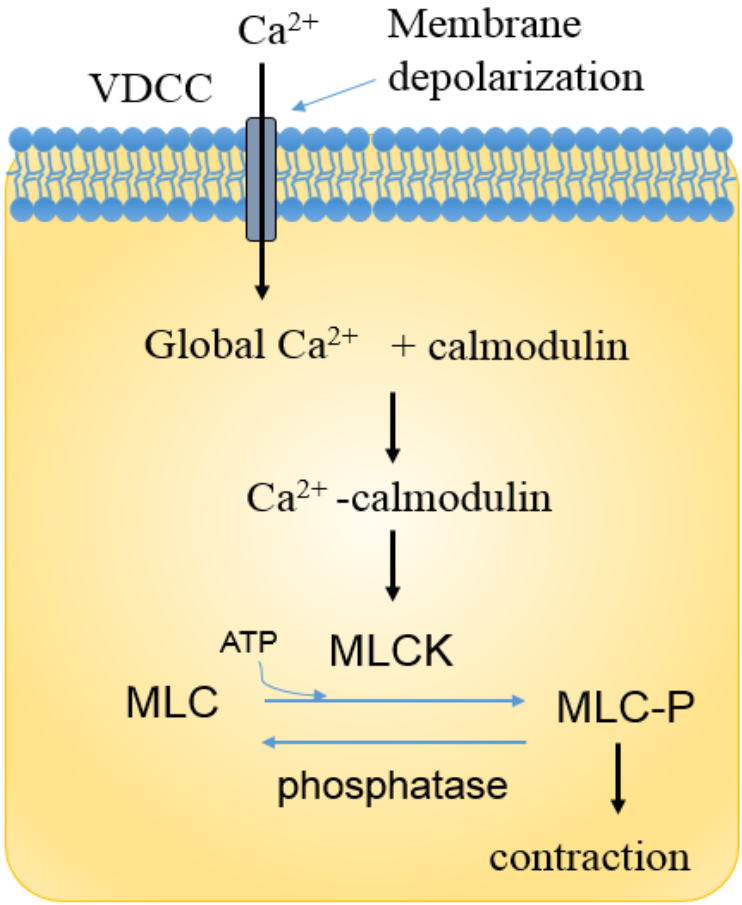


**Figure 1-1. Schematic diagram of smooth muscle contraction.**

*Notes:* Membrane depolarization activates voltage dependent $Ca^{2+}$ channels. Voltage dependent $Ca^{2+}$ channels (VDCC) induce $Ca^{2+}$ entry and increase global $Ca^{2+}$. $Ca^{2+}$ binds to calmodulin which forms $Ca^{2+}$-calmodulin complex. $Ca^{2+}$-calmodulin complex further stimulates myosin light chain kinase (MLCK). MLCK phosphorylates light chains in myosin heads. Myosin slides along actin which creates muscle contraction.

in outward efflux of potassium which shifts the membrane potentials more negatively. So BK channel regulates myocyte membrane potential thereby arterial contractility.

## BK channel function

Membrane depolarization elevates intracellular $Ca^{2+}$ which activates BK channels. Membrane depolarization opens voltage dependent $Ca^{2+}$ channel which results in $Ca^{2+}$ influx into the cell. (Jaggar, Porter et al. 2000) The intracellular $Ca^{2+}$ rise is pumped into sarcoplasmic reticulum by sarcoplasmic reticulum $Ca^{2+}$ ATPase. (Jaggar, Porter et al. 2000) Sarcoplasmic reticulum $Ca^{2+}$ load activates the ryanodine receptors, which generate localized $Ca^{2+}$ sparks that activate BK channel. (Jaggar, Porter et al. 2000) Activated BK channels would induce hyperpolarization which inhibits voltage dependent $Ca^{2+}$ channel and reduces $Ca^{2+}$ entry. (Jaggar, Porter et al. 2000) So BK channels have a profound negative feedback on vascular contractility.

## BKα subunit

BK channel is comprised of four pore-forming α subunits. (Hu and Zhang 2012, Yang, Zhang et al. 2015) It was first found in the Drosophila slowpoke mutant in 1980s. (Elkins, Ganetzky et al. 1986) The BKα subunits are encoded by KCNMA1 gene (a.k.a SLO). (Hu and Zhang 2012, Yang, Zhang et al. 2015) The BKα subunit contains seven transmembrane helices (S0-S7) which form a voltage sensor domain and a pore gate domain as well as a large COOH terminal region. (Yang, Zhang et al. 2015) Transmembrane segment S0 forms an extracellular N terminus. (Yang, Zhang et al. 2015) S1-S4 helices form the primary voltage sensor domain (VSD). (Yang, Zhang et al. 2015) Membrane depolarization induces conformational change in the voltage sensor domain by twisting of S4 segment upwardly. (Yang, Zhang et al. 2015) Charged arginine in S4 domain contributes to the gating charge, specifically Arg213, Asp153, Arg167 and Asp186, which are voltage sensing. (Stefani, Ottolia et al. 1997, Ma, Lou et al. 2006) S5-S6 helices form the pore gate domain. (Jiang, Pico et al. 2001) The cytosolic tail domain includes two regulators of conductance for $K^+$ (RCK domain). (Jiang, Pico et al. 2001) The second RCK domain contains $Ca^{2+}$ bowl which is a high affinity binding site for $Ca^{2+}$. (Jiang, Lee et al. 2002) Four RCK domains are assembled to form a gating ring which can change its conformation upon $Ca^{2+}$ binding. (Hu and Zhang 2012) A heme binding site locates at a region between two RCK domains (Hou, Heinemann et al. 2009), which inhibits channel activity in the presence of heme. (Tang, Santarelli et al. 2004) The voltage sensor domains transduce membrane potential changes which are coupled to the gate domain. (Hu and Zhang 2012) The BK channel activity is subject to regulation by a great variety of biological factors such as nitric oxide (NO), carbon monoxide (CO), steroid hormones, reactive oxygen species (Amberg, Bonev et al.), protons ($H^+$), heme, lipids and metabolites (Yang, Zhang et al. 2015).

## β subunit

BK channels auxiliary β subunits consist of two transmembrane domains connected by a long extracellular loop, which can co-assemble with α subunits. (Hu and Zhang 2012) Four types of β subunits (β1-β4) of BK channel have been identified and β1 subunit is mainly expressed in the smooth muscle cells, β4 is expressed in neural tissue. (Kyle and Braun 2014) Voltage sensor domain is stabilized in its active form by β1, β2 and β4 subunits (Contreras, Neely et al. 2012). The β2 and some variants of β3 subunits induce inactivation of BK channel through N-termini. (Wallner, Meera et al. 1999) (Uebele, Lagrutta et al. 2000) Up to four auxiliary β1 subunits can co-assemble with the tetrameric BKα subunits, particularly interact with N-terminal S0-S2 segment which regulate the gating property of BK channels. (Hu and Zhang 2012) The β1 subunits have been shown to elevate $Ca^{2+}$ sensitivity and modulate gating properties. (Wu and Marx 2010) The β subunits also enhance the coupling of ryanodine receptor mediated $Ca^{2+}$ sparks to spontaneous transient outward currents (STOCs) which subsequently reduce $Ca^{2+}$ influx and result in relaxation. (Jaggar, Porter et al. 2000) The studies of knock-out β1 subunits mice show that a decrease in the calcium sensitivity of BK channels and a reduction in calcium sparks so that the vascular relaxation mechanism is significantly impaired and mean arterial blood pressure is significantly elevated. (Brenner, Perez et al. 2000) Mutation of G352A in β1 gene of BK channels has been shown to associate with low prevalence of diastolic hypertension in an epidemiological study. (Fernandez-Fernandez, Tomas et al. 2004) Expressing a mutant form, β1 E65K, together with wild type β1 subunits in HEK 293 cells, enhanced $Ca^{2+}$ sensitivity of BK channels. (Fernandez-Fernandez, Tomas et al. 2004) So β1 subunits are essential for regulation of BK channel activity to control the vascular contractility.

## $Ca^{2+}$ sparks

$Ca^{2+}$ sparks represent rapid spatially localized $Ca^{2+}$ rise released from Ryanodine-sensitive $Ca^{2+}$ channels (RyR) of sarcoplasmic reticulum (Kilts, Gerhardt et al.) (Kilts, Gerhardt et al.). Smooth muscle SRs are within 20 nm to cell membrane, indicating a direct communication between SR and cell membrane BK channels. (Devine, Somlyo et al. 1972) RyR channels are first found by activation of a plant alkaloid ryanodine in striated muscle of insect. (Sutko and Airey 1996) Three RyR channels have been identified as RyR1, RyR2 and RyR3 which express in skeletal muscle, cardiac muscle and brain respectively. (Jaggar, Porter et al. 2000) RyR receptors generate micromolar elevation of $Ca^{2+}$ with a rise time of ~20 ms which activates nearby BK channels. (Bonev and Nelson 1993) The frequency of $Ca^{2+}$ sparks is about 1 Hz in resting potential of rat cerebral artery myocytes. (Jaggar, Porter et al. 2000) Fill width at half maximal amplitude of $Ca^{2+}$ sparks is around 2~3 um. (Jaggar, Stevenson et al. 1998) $Ca^{2+}$ sparks have no effect on contractility directly due to their limited spatial reach and no alteration of global $Ca^{2+}$ concentration. (Jaggar, Porter et al. 2000) BK channel has a low $Ca^{2+}$ affinity which could not be stimulated by global $Ca^{2+}$. The decay of $Ca^{2+}$ sparks is caused by diffusion, uptake $Ca^{2+}$ into SR and extrusion. (Santana, Kranias et al. 1997) Elevation of cGMP or cAMP increased $Ca^{2+}$ sparks frequency and subsequently activates BK channel activity.

(Jaggar, Wellman et al. 1998, Porter, Bonev et al. 1998) Phospholamban dissociated from SR $Ca^{2+}$ -ATPase after phosphorylation by PKG or PKA, resulting in an increase in SR $Ca^{2+}$ load. (Santana, Kranias et al. 1997) (ZhuGe, Tuft et al. 1999) Activation of PKC reduced $Ca^{2+}$ sparks and subsequently STOC frequency and amplitude in myocytes from cerebral arteries. (Bonev, Jaggar et al. 1997)

**γ subunits**

A leucine-rich repeat containing subunits (LRRCs), referred to as γ subunits have been identified in the BKα pull-down components which can regulate BK channel activity. (Evanson, Bannister et al. 2014) The ~35kDa LRRC protein is classified as another type of BK channel auxiliary subunit due to its functionally and structurally distinction. (Evanson, Bannister et al. 2014) The LRR domain contains repeating 20-29 residue leucine rich sequence. (Evanson, Bannister et al. 2014) LRRC proteins are characterized by the consensus sequence: LXXLXLXX $^{N}/_{C}$ XL, where L is leucine, isoleucine or phenylalanine and X is any amino acid. (Yan and Aldrich 2012) LRRC 26, 38, 52 and 55 have been found to increase voltage sensitivity of BK channels and shift to hyperpolarization direction. (Yan and Aldrich 2012) LRCC26 subunits induce a large negative voltage shift (~ -140mV) in BK channel activation. (Yan and Aldrich 2012) LRCC26 has been found to be expressed and associated with BKα channels in cerebral arteries. (Evanson, Bannister et al. 2014) LRCC26 increases voltage- and $Ca^{2+}$ sensitivity of BK channel. (Evanson, Bannister et al. 2014)

**Post translational modification**

BK channels are subjective to extensive post-translational modifications which significantly change the property of channel function. (Kyle and Braun 2014) Ser/Thr/Tyr residues in pore-forming α subunit are modified by protein kinases or protein phosphatases. (Kyle and Braun 2014) Adenylyl cyclase catalyzes ATP into cyclic AMP. Protein kinase A (PKA) contains two catalytic domains which associate with two regulatory domains. cAMP binds to regulatory subunits of PKA which in turn releases catalytic domain from PKA. PKA-mediated phosphorylation of BK channel enhances BK channel activity. Ser873 in BK is essential for an increase in PKA-mediated BK channel activity. (Nara, Dhulipala et al. 1998)

Many studies showed that BK channels are extensively regulated by various protein kinase phosphorylation. Regarding of protein kinase G (PKG) pathway, nitric oxide (NO) stimulates guanylyl cyclase, which results in synthesis of cGMP from GTP. Sodium nitroprusside releases nitric oxide. PKG-mediated mediated phosphorylation of BK channel also increases BK channel activity. (Robertson, Schubert et al. 1993) It has been reported that Ser691, 873 and 1111-1112 in the BK C-terminus are modified by NO/cGMP/PKG signaling pathway to enhance channel activity (Kyle, Hurst et al. 2013). Furthermore, the cAMP-dependent protein kinase (PKA) activates the BK channel by increasing $Ca^{2+}$ sensitivity of the channel (White, Kryman et al. 2000). On the contrary,

protein kinase C inhibits BK channels in smooth muscle (Zhou, Wulfsen et al. 2010). 20-hydroxyeicosatetraenoic acid, a protein kinase C activator, has been shown to inhibit the whole cell potassium current in the cerebral vascular smooth muscle (Lange, Gebremedhin et al. 1997). The active diacylglycerol analog has also been shown to reduce the potassium current by 70% in rat tail artery smooth muscle cells (Schubert, Noack et al. 1999). Phosphorylation of Ser695 and Ser1151 by PKC activation has been shown to decrease BK channel activity in myocytes. (Zhou, Wulfsen et al. 2010). Besides of Ser/Thr phosphorylation, BK channels are also enhanced by Src kinases and Pyk-2 which phosphorylate Tyr in BKα subunit. (Ling 2000, Yang et al 2012) Tyr766Phe in the C-terminus of BK subunit inhibits c-Src medicated phosphorylation. (Ling 2000, Yang et al 2012)

## Endothelin

Arterial contractility is modulated by both vasoconstrictor and vasodilator stimuli, leading to change in regional organ blood flow and systemic blood pressure. Vasoconstrictors, including endothelin-1 (ET-1), decrease arterial diameter, which reduce organ blood flow and elevate blood pressure. (Yanagisawa, Kurihara et al. 1988) In contrast, vasodilators, including nitric oxide (NO), increase arterial diameter to enhance organ blood flow as well as reduce blood pressure. (Archer, Huang et al. 1994) Vasoregulatory stimuli modulate the activity of ion channels expressed in cells of the vascular wall, resulting in a change in membrane potential, a key determinant of contractility. (Nelson, Patlak et al. 1990) Many different ion channels expressed in arterial smooth muscle cells (myocytes) regulate membrane potential, which modulate $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels thereby change intracellular $Ca^{2+}$ concentration. (Jaggar, Porter et al. 2000)

Endothelin is a twenty-one amino acid peptide discovered by Yanagisawa in 1988 (Yanagisawa, Kurihara et al. 1988). It is a potent vasoconstrictor that has three isoforms, ET-1 ET-2, ET-3 (Inoue, Yanagisawa et al. 1989). Endothelin-1 is mainly generated by the endothelium. ET-1 acts on its receptors of smooth muscle cells via autocrine or paracrine pathway. (Ivey, Osman et al. 2008, Horinouchi, Terada et al. 2013) Endothelin receptors consist of two subtypes: $ET_AR$ and $ET_BR$, both belong to GPCR superfamily, which. (Ivey, Osman et al. 2008) $ET_AR$ is highly expressed in the vascular smooth muscle cell that contributes to ET-1 induced contraction and proliferation. (Ivey, Osman et al. 2008) $ET_B$ receptors have many functions, such as clearance of ET-1, release of NO and prostaglandin, inhibition of endothelial cell apoptosis. (Ivey, Osman et al. 2008) Endothelin receptors mediate vascular contractility, cell proliferation and differentiation. (Ivey, Osman et al. 2008)

ET-1 activates at least three signaling pathways in vascular smooth muscle cell, including PKC, MAPK and PI3K/PKB pathways. (Hilgers and Webb 2005) The binding of ET-1 to the $ET_A$ receptor activates Gq protein. (Woodsome, Polzin et al. 2006) Gq protein leads to activation of membrane bound enzyme phosphoinositide specific phospholipase C (PLC), which then hydrolyzes the membrane phospholipid

phosphatidylinositol-4,5-bisphosphate ($PIP_2$) to two second messengers hydrophobic diacylglycerol (DAG) and inositol-1,4,5-trisphosphate ($IP_3$) (Hilgers and Webb 2005, Woodsome, Polzin et al. 2006). Binding of $IP_3$ to the $IP_3$ receptor triggers release of $Ca^{2+}$ from sarcoplasmic reticulum. (Lesh, Nixon et al. 1998) The RyR, localized on the sarcoplasmic reticulum, facilitates $IP_3$ to bind $IP_3$ receptor. (Lesh, Nixon et al. 1998) RyR-mediated $Ca^{2+}$ channels lead to a rapid increase in $Ca^{2+}$ concentration. (Lesh, Nixon et al. 1998) $Ca^{2+}$ binds calmodulin to form a $Ca^{2+}$ -calmodulin complex, which activates myosin light chain kinase (MLCK) to phosphorylate light chain of myosin. It enables interaction of myosin with actin to generate contraction. DAG, together with $Ca^{2+}$, activates the protein kinase C. (Hilgers and Webb 2005) DAG medicates vasocontricton via activation of PKC. PKCα and PCKβ are dependent upon intracellular $Ca^{2+}$ and activation of DAG. (Hilgers and Webb 2005) PKCε is dependent only on DAG. PKC directly phosphorylates MLCK which in turn phosphorylates MLC. (Hilgers and Webb 2005) The phosphorylated MLC facilitates the interaction between myosin and actin filaments. Myosin cross bridges with actin resulting in contraction. (Hilgers and Webb 2005)

ET-1 receptor also activates Raf and mitogen-activated protein kinases (MAPK) cascade through phosphorylation of PKC (Moodie, Willumsen et al. 1993). PKC and calmodulin stimulate Ras which recruits Raf. (Moodie, Willumsen et al. 1993) Raf phosphorylates MAPK/ERK kinase which in turn phosphorylates extracellular signal-regulated kinases (ERK) that controls vascular smooth muscle cell survival, differentiation and inflammation. (Moodie, Willumsen et al. 1993) Phosphatidylinositol-3 ($PIP_3$) kinase is also an effector of ET-1 action (Shi-Wen, Chen et al. 2004), which converts phosphatidylinositol 4,5- bisphosphate to $PIP_3$ through phosphatidylinositol 3-kinase (PI3K). $PIP_3$ acts as an second messenger to activate protein kinase B (PKB), which is involved in protein synthesis and cell survival (Proud 2004).

## Rab Protein

Rab protein is a key regulator of various membrane trafficking events which belongs to the small GTPase family. (Pfeffer 2005) The Rab family comprises more than 70 members, each of which has specific intracellular localization and controls a determined vesicular transport pathway, such as endocytosis, protein trafficking, endosome fusion, exocytosis. (Pfeffer 2005) By far, only a small fraction of Rab protein functions have been identified. Rab proteins facilitate transport along the cytoskeleton and participate in fusion and docking. Rab proteins cycle between an active and an inactive state (Pfeffer 2005). Binding of GTP to Rab stimulates its function whereas association with GDP inactivates it. (Pfeffer 2005) Guanine nucleotide exchange factors (GEF) exchange GDP for GTP to generate the active Rab GTP form, whereas GTPase activating proteins (GAP) generate the inactive Rab-GDP form. (Pfeffer 2005)

At the beginning of internalization, ligands are sequestered into clathrin coated pits. Rab5 is localized to the membrane, early endosomes and clathrin coated vesicles. Endocytosis of internalized extracellular molecules is initiated by Rab5 (Christoforidis,

McBride et al. 1999), which directs plasma membrane derived vesicles to Rab4 positive early endosomes. Molecules can be then recycled back to the plasma membrane from the early endosome via two pathways: a fast direct route that depends on Rab4, and a slow route via Rab11 positive late recycling endosomes. (Chen, Feng et al. 1998) Rab11 mediates intracellular vesicle trafficking that delivers plasma membrane proteins from the Golgi complex to the cell surface along actin filaments (Chen, Feng et al. 1998). Rab4 controls the rapid recycling of cargo proteins directly back to the cell membrane (D'Souza, Semus et al. 2014). BKα protein is trafficked in Rab4A-mediated endosomes to the plasma membrane in myocytes. (Leo, Bulley et al. 2015) Rab25 protein has been found to regulate surface expression of $Ca_v$ 1.2 channel in arterial myocytes. (Bannister, Bulley et al. 2016)

## Stroke-prone Spontaneously Hypertensive Rat

The stroke-prone spontaneously hypertensive rat (SP-SHR) is a typical genetic model for studying hypertension and cerebral stroke. SP-SHRs were bred from a strain of spontaneously hypertensive rats in 1963 by Okamoto et al. (Okamoto and Aoki 1963) The third generations of hypertensive rats developed 80% incidence of stroke as well as hypertension (180-200 mmHg). (Okamoto and Aoki 1963) The pathological finding of cerebral stroke in SP-SHR does not lie on atherosclerosis which results in impaired brain perfusion and cerebral infarction. (Nabika, Cui et al. 2004) Instead, the pathophysiological mechanism of SP-SHRs might be that malignant hypertension leads to brain edema and lacunar infarction. (Nabika, Cui et al. 2004)

It has been reported that lower expression of β1 protein reduced the $Ca^{2+}$ sensitivity of BK channels in arterial smooth muscle cells of SP-SHRs compared with SD rats. (Amberg and Santana 2003) This could explain why mean blood pressure increased in SP-SHR compared with SD rats. But the expression of β1 protein did not have statistically difference between WKY and SP-SHR. (Amberg and Santana 2003) Previous studies indicated that NADPH oxidase is elevated in various tissue of SP-SHRs. (Chabrashvili, Tojo et al. 2002, Panico, Luo et al. 2009) Superoxide was produced by NADPH oxidase (NOX) in the kidney of SP-SHR. (Chabrashvili, Tojo et al. 2002, Panico, Luo et al. 2009) Elevated expression of NADPH oxidase 3 was also found in the cerebrum of SP-SHRs. (Nozoe, Hirooka et al. 2007, Michihara, Oda et al. 2016) Increased NADPH oxidase generates reactive oxygen species in tissues of SP-SHRs. (Amberg, Bonev et al.) Elevated reactive oxygen species (Amberg, Bonev et al) degrade free NO into $ONOO^-$, therefore decrease NO level. Vascular smooth muscle cells of rats exposed to oxidative stress ($H_2O_2$) results in dose- and time- dependent activation of PKC. (Li, Maasch et al. 1999) Imidapril, an angiotensin converting enzyme inhibitor, prevented the incidence of stoke in SP-SHRs. (Ogiku, Sumikawa et al. 1993) So increased reactive oxygen species may be one of underlying mechanisms to induce hypertension in SP-SHRs.

# CHAPTER 2. HYPOTHESIS

## Goal

The large-conductance Calcium ($Ca^{2+}$)-activated potassium channel (BK channel) plays a vital role in maintaining the dynamic regulation between vasoconstriction and vasodilation of the vascular smooth muscle. The stimulators of BK channel include membrane depolarization and elevation of intracellular $Ca^{2+}$. Previous studies have focused on the open probability and single channel current of BK channels regulated by protein kinases. But the mechanism how protein kinases regulate the number of functional channels on the cell membrane is not well understood. Previous common concept is that all channel subunits are assembled completely before trafficked to cell surface. Previous data shows that regulatory β1 subunits resided predominantly inside the cell and were rapidly trafficked to cell membrane and associated with α subunits by activation of Protein Kinase G (PKG) or Protein Kinase A (PKA) (Leo, Bannister et al. 2014). However, the regulation of β1 subunit trafficking by Protein Kinase C (PKC) pathway is not known. We hypothesize that activation of PKC can reduce PKG-induced β1 subunits trafficking to cell membrane. The following aims are proposed to investigate the mechanism.

## Specific Aims

### Specific aim 1

The membrane current (I) generated by a population of ion channels, such as BK, is determined by their open probability (Po), the number of channels (N) and single channel current (i).(Leo, Bannister et al. 2014) Recent evidence indicates that in resting arterial myocytes, only a small proportion of total β1 subunits are present at the plasma membrane, with most β1 protein located intracellularly within Rab11A-positive recycling endosomes.(Leo, Bannister et al. 2014) NO stimulates rapid (<1 min) protein kinase G (PKG)-mediated surface trafficking of intracellular β1 subunits, which associate with BKα to increase apparent $Ca^{2+}$-sensitivity, thereby elevating open probability. (Leo, Bannister et al. 2014) Stimulated β1 subunit trafficking in myocytes is a principal mechanism by which NO promotes cerebral vasodilation. (Leo, Bannister et al. 2014) Membrane depolarization also activates BK channels by stimulating Rho kinase-mediated β1 subunit trafficking in human and rat arterial myocytes. (Leo, Zhai et al. 2017) It is unclear whether vasoconstrictors inhibit BK channels and stimulate contraction by reducing the surface abundance of β1 subunits and their association with BKα. If such mechanisms exist, it would be important to determine the processes by which vasoconstrictors decrease surface levels of β1.

Previous data shows that sodium nitroprusside (SNP), a vasodilator that releases nitric oxide to activate PKG pathway, increased β1 subunits trafficking to surface more

than two-fold. We plan to focus on endothelin-1 (ET-1), which is a potent vasoconstrictor synthesized by endothelial cells. We hypothesize that ET-1 can reduce SNP-induced β1 subunits trafficking to cell membrane. Localization of two subunits on the surface membrane or in the intracellular compartment can be determined by surface biotinylation and western blotting. We will also evaluate the localization and spatial proximity between α and β subunits by immunoFRET microscopy.

ET-1 activates several signaling pathways in vascular smooth muscle cell, including PKC, MAPK and PKB pathways. We hypothesize that endothelin-1 reduces SNP-induced β1 subunits trafficking to cell membrane via PKC pathway. Bisindolylmaleimide-1 (BIM-1) acts as a competitive inhibitor for the ATP binding site of PKC and shows high selectivity for PKCα-, β1-, β2-, γ-, δ-, and ε-isozymes. We plan to test if BIM-1 can abolish the reduction of β1 subunits trafficking to surface by inhibiting PKC pathway. Surface biotinylation and immunoFRET microscopy will be performed to test the hypothesis.

To evaluate the effects of vasoconstrictor on the physiological function of BK channels, we will perform inside-out patch clamp to determine whether endothelin-1 can decrease SNP-induced elevation of BK channel open probability via PKC pathway. We also plan to perform perforated patch clamp to determine whether endothelin-1 can reduce the frequency and amplitude of spontaneous transient outward currents (STOCs) of BK channel via PKC pathway.

Physiological functions of endothelin-1 can be measured by using cannulated arteries pressurized at 60 mmHg. We plan to perform pressurized cerebral artery diameter measurements to examine whether endothelin-1 can inhibit SNP-induced vasodilation by inhibiting β1 subunits trafficking to cell membrane. We also expect that BIM-1 can decease endothelin-1 induced vasoconstriction.

**Specific aim 2**

Previous data shows that Rab11A was co-localized with β1 subunits in the highly mobile Rab11A positive recycling endosome in smooth muscle cells. The smooth muscle cells transfected with dominant negative Rab11A S25N failed to respond to PKG stimulation. We hypothesize that PKC may phosphorylate different sites of Rab11A to inhibit anterograde trafficking of β1-containing Rab11A recycling endosome. Bioinformatics analysis displays that there are 17 probable phosphorylation sites in Rab11A, of which Ser177 has the highest probability (Pavarotti, Capmany et al. 2012). We hypothesize that PKC phosphorylates Ser177 which inhibits Rab11A function of regulating trafficking.

We plan to transfect smooth muscle cells with dominant negative Rab11A S177A and test whether PKC fails to inhibit Rab11A S177A endosome anterograde trafficking. We also plan to inside-out patch clamp to test whether Rab11A S177A alters ET-1 inhibition of BK channel activity. Furthermore, we plan to perform pressurized cerebral

artery diameter measurement to examine whether Rab11A S177A modulate ET-1-induced vasoconstriction.

**Specific aim 3**

BKα and β1 surface and total protein, cellular distribution, spatial proximity, and association (co-IP) will be measured in SP-SHR arteries. Regulation of these BKα and β1 properties by vasodilators (NO donors) and membrane potential (by elevating $[K^+]_o$) will be examined. Pressurized hypertensive rat cerebral arteries are depolarized. Intracellular signaling pathways will be studied to identify pathological changes in mechanisms that control BKα and β1 subunit surface expression. Rab11A protein will be measured. Immunofluorescence and immune-FRET will measure colocalization and spatial proximity of BKα and β1 subunits to each other, Rab proteins. If expression of a Rab protein is reduced, we will overexpress that wild-type Rab in arteries and determine if pathological BKα and/or β1 trafficking or degradation is reversed.

BK channel properties, and regulation by lithocholate will be measured in myocytes of WKY and hypertensive rats. Experiments would be investigated altered intracellular signaling pathways and Rab proteins that underlie dysfunctional regulation of BK channel subunits by vasodilators and membrane depolarization. Endothelium-denuded arteries will be studied. Responses to lithocholate and following exposure to NO donors will be measured.

# CHAPTER 3. METHODOLOGY

## Animal Subjects

### Animal use

Male Sprague-Dawley rats (7-8 weeks) or male WKY rats (6 weeks), male SP-SHR (6 weeks) were purchased from Harlan-Teklad Laboratories (Ewing, IL). All experiments were conducted ex vivo. We conducted the research in accordance with the Guide for Care and Use of Laboratory Animals. The Animal Care and Use Committee reviewed and approved all animal protocols at the University of Tennessee Health Science Center.

### Euthanasia

All euthanasia protocols were approved by IACUC. Male Sprague-Dawley rats (7-8 weeks) were knocked down with carbon dioxide directly introduced into a special chamber. Rats were euthanized with intraperitoneal injection of sodium pentobarbital (150mg/kg) (Vortech Pharmaceuticals, Dearborn, MI). New needles and syringes were used each time to avoid additional trauma. All efforts were done to reduce distress to rats during the euthanasia procedure. The method was followed according to euthanasia recommendations from the American Veterinary Medical Association Panel. Sodium pentobarbital was used to induce sedation, followed by unconsciousness. Animal euthanasia was confirmed by loss of corneal reflex, minimum movement to skin pinch, or cessation of respiration. After confirmation, rats were euthanized by decapitation.

## Tissue Preparation

The brain was removed, cleaned and placed in ice-cold HEPES-buffered physiological saline solution containing (in mmol/L): 134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4). Cerebral arteries (anterior cerebral, middle cerebral, posterior cerebral, cerebellar, basilar) were dissected from the brain. Cerebral arteries were carefully cleaned from brain tissue and connective tissue under microscope (Nikon), preventing the arteries from any stretch or damage. The cleaned arteries were placed in iced cold filtered PSS, which was ready for use.

## Isolation of Arterial Smooth Muscle Cells

### Preparation of solutions used for smooth muscle cell isolation

Isolated cerebral arteries were placed for in a $Ca^{2+}$- free whole cell buffer solution containing (in mM): 55 NaCl, 5.6 KCl, 80 sodium glutamate, 2 $MgCl_2$, 10 HEPES, and 10 glucose (with pH adjusted to 7.4 with NaOH). Three glass vials were rinsed and washed with distilled water. Papain (0.7 mg/ml, Sigma), dithioerythreitol (1mg/ml) and bovine serum albumin (BSA) (1 mg/ml) were dissolved in $Ca^{2+}$- free solution. Collagenases F (0.7 mg/ml) and collagenase H (0.3 mg/ml) were added to $Ca^{2+}$- free solution. Before isolation, collagenase solution was supplemented with $CaCl_2$ (100 μM). Papain and collagenase solutions in vials were warmed in 37°C water bath for 10 minutes before transferring cerebral arteries into the vials.

### Freshly dissociated cerebral arteries

When papain solution in vial was warmed, cerebral arteries were placed into the vial and incubated for 12.5 minutes in 37°C water bath. Next, the arteries were promptly transferred to pre-warmed collagenase solution and incubated for 7 minutes in 37°C water bath. Incubation time was adjusted to 15 mins for papain and 9 mins for collagenase when more myocytes were needed to be isolated from cerebral arteries. After collagenase digestion, cerebral arteries were subsequently transferred in a vial and washed by ice-cold $Ca^{2+}$-free solution for three times to stop enzyme digestion. The rinsed arteries were transferred to another vial containing $Ca^{2+}$-free solution and put on ice to settle down for 10 min. About 1ml $Ca^{2+}$-free whole cell solution was added into vial which contained digested cerebral arteries. A special glass pipette was used to gently triturate the arteries up and down for ten times. No air bubbles was introduced into the solution otherwise the bubbles could harm the myocytes. Myocytes were gently dissociated from the arteries during the trituration process. Myocytes were ready for use within next 8 hours.

## Arterial Surface Biotinylation

### Artery biotynylation

Intact arteries were incubated in low binding vial containing 1 mg/ml EZ-Link Sulfo-NHS-LC-LC-Biotin and 1 mg/ml EZ-Link Maleimide-$PEG_2$-Biotin reagents (Pierce) HEPES-buffered physiological saline solution containing for 1h at room temperature. Sodium nitroprusside (10 μM) was added at last 10 mins during the incubation. Endothelin-1 (10 nM) or BIM (20 μM) was added at the beginning of biotinylation at each condition. The vial containing intact arteries was centrifuged for 2 mins at 6.0 rpm. The PSS containing biotin was removed by pipette. Free biotin was

quenched by washing the arteries in PBS with 100 mmol/L glycine for 15min. Arteries were then washed in PBS to remove any residual glycine solution for 15min. Biotinylated arteries were homogenized in lysis buffer containing Tris 50mM, NaCl 150mM, EDTA 500mM, 1% Triton X-100, 0.1% SDS with protease inhibitors and phosphatase inhibitors.

**Protein concentration estimation**

5 μL protein sample was added onto nitrocellulose membrane (Bio-Rad, CA) and dry before use. The nitrocellulose membrane was incubated with Amido black solution for 2 mins. Then, the membrane was washed by distilled water for 2mins. The membrane was then washed by the destained solution for 2 mins to wash off any residue Amido black. The membrane containing stained protein sample in glass tube was added with 1 mL elute solution and incubated for 25min to dissolve the Amido black. The elute solution was measured by spectrometer. The result was applied into the standard curve of protein concentration.

**Protein pulldown**

Total protein was pulled down with avidin (1:1.2, Thermos Scientific) for 1h in the cold room. After pulldown, the sample was centrifuged at 13,000g for 2 mins. Biotinylated proteins were bound to the avidin beads and the supernatant contained the nonbiotinylated (intracellular) protein fraction. The beads were washed 3 times with PBS, and Laemmli buffer was added to both the beads and intracellular protein lysate. Samples were then boiled for 3 minutes. Biotinylated surface proteins were eluted from the avidin beads. Both protein fractions were analyzed using Western blotting.

## Western Blotting

Protein lysates were heated with 1× or 5× Laemmli buffer (Bio-Rad) for 3 mins. Protein was separated on 7.5% or 12% SDS-PAGE polyacrylamide gels. Molecular weight markers (Bio-Rad, Hercules, CA) were used in one lane to monitor protein progress on the gel. Gel was run in a chamber containing SDS running buffer (containing 12g Tris, 57.6g Glycine and 3g SDS into total 4L distilled water) at 150 V for ~1h. The run was stopped when the lowest marker reached the bottom of gel.

The gel was placed with nitrocellulose membrane (Bio-Rad, Hercules, CA) between two sponges and two filter paper in a binding cassette as a “transfer sandwich”. No bubbles were introduced between gel and membrane otherwise it would cause bald spots. The cassette was then placed into a chamber containing transferring buffer (containing 12g Tris, 57.6g Glycine and 800 ml methanol into total 4L distilled water). Proteins were transferred for 1.5h at 100 V using a Mini Trans Blot Cell (Bio-Rad, Hercules, CA) in a cold room. Protein was transferred onto blots.

Blots were blocked with 5% nonfat dry milk or 5% BSA in Tris-buffered solution (TBS) added with 0.1% Tween-20 (TBS-T) at room temperature for 1h. Membranes were then probed with mouse monoclonal anti-BKα (1:500 dilution, Neuromab, UC Davis), rabbit polyclonal anti-BKβ1 (1:500, Abcam), rabbit monoclonal anti-rab11A (1:500, Cell Signaling), rabbit polyclonal anti-phosphoserine (1:500, Millipore) or mouse monoclonal anti-active rab11 (1:500, NewEast Biosciences) antibodies overnight at 4°C.

The blots were incubated with primary antibodies overnight in cold room. Then the blots were washed with TBS-T for 6 times 10min each. Then the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (mouse or rabbit) in 5% nonfat dry milk for 1h at room temperature. After that, the blots were washed 6 times with TBS-T. The membranes were exposed to reagents from a chemiluminescent detection kit (supersignal west femto pierce, Thermo Scientific). The blots were imaged with a Kodak In Vivo F Pro Imaging System or Bio-Rad ChemiDoc Touch imaging system. Band intensity was quantified using Quantity One software (Bio-Rad). Membranes were developed using different exposure time in order to optimize band intensity.

Using Restore Western Blot Stripping Buffer (Thermo), the blots were stripped for 5-10 mins at room temperature. The blots were then washed with TBS-T twice and re-blocked with 5% nonfat dry milk in Tris-buffered solution for 1h. The blots were then ready for re-probing with other indicated antibodies. Band intensity was quantified using Quantity one software (Bio-Rad, Hercules, CA). The band density was calculated by placing a rectangular box around the band, which then subtracted with background density with the same size of the box. Average band density was calculated from images of different exposure time. The band density of a specific protein was normalized with that of the loading control protein actin or cytochrome C.

**Immunofluorescence and Immuno-FRET Microscopy**

Isolated myocytes were exposed to agents and then were plated on poly-L-lysine-coated coverslips. Cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked with 5% bovine serum albumin (BSA) and then incubated with mouse monoclonal anti-BKα (Neuromab, UC Davis) and rabbit polyclonal anti-BKβ1 (Abcam) antibodies overnight at 4°C. After washing with PBS, cells were incubated for one hour with Alexa 546- or Alexa 488-conjugated secondary antibodies (1:100 dilution; Life Technologies). After washing, coverslips were mounted onto slides with 1:1 glycerol: PBS media. Fluorescence images were acquired using a laser-scanning confocal microscope (LSM5 Pascal; Carl Zeiss). Alexa 488 and Alexa 546 were excited at 488 and 543 nm, and emission was collected at 505-530 and ≥560 nm. For N-FRET analysis, images were background-subtracted and N-FRET calculated on a pixel-by-pixel basis using the Xia method (Xia and Liu 2001) and Zeiss LSM FRET Macro tool (Version 2.5).

## Transformation

### Transformation of competent cells

Competent GC10 bacterial cell (50 µg, Sigma-Aldrich, St. Louis, MO) were used for transformation. Vectors (>20ng) were added to GC10 cells and incubated on ice for 15 mins. Competent cells were then heat shocked in a water bath at 42ºC for 45 seconds. The cell membrane was permeabilized by the heat shock which allowed vectors enter the cells. After heat shock, the cells were placed on the ice. Transformed cells were plated onto ampicillin (100 µg/ml) resistant LB plates beside Bunsen burner. Agar plates were then incubated at 37 °C overnight. Bacteria which have expressed vectors containing ampicillin resistant gene grew in colonies on agar plates.

### DNA Maxiprep

A single bacterial colony which expressed vectors was inoculated into LB broth supplemented with ampicillin (100 µg/ml) and incubated at 37°C at 200 rpm overnight. Plasmid cDNA was extracted and purified from the bacteria using Qiagen Maxiprep HiSpeed Plasmid Purification kit (Qiagen, Valencia, CA).

LB broth was made and autoclaved in a 1L flask. After LB broth was cool, 50 mg/ml ampicillin was added into the LB broth. A bacterial colony was picked and put into LB broth besides the Bunsen burner. The flask was incubated in 37ºC incubator overnight. On the next day, LB broth was put into plastic centrifuge bottle and then centrifuged for 15 mins at 5000 rpm. The supernatant was gently poured off and bacterial pellet was re-suspend in 10 ml Buffer P1 on ice. The re-suspended pellet solution was transferred to a plastic centrifuge tube. 10 ml Buffer P2 was added into tube and mixed gently. The mixed solution was incubated at room temperature for 5 minutes. Then 10 ml of cold Buffer P3 was added and mixed immediately but gently by inversion. The solution was incubated on ice for 20 minutes and then centrifuged for 30 mins at 12,000 rpm at 4ºC. During the last 10 mins of the spin, Qiagen-tip 500 column was equilibrated by adding 10 ml Buffer QBT. The supernatant was decanted in Falcon 50ml tubes. Decanted supernatant was added to column. The column was washed twice with 30 ml Buffer QC. The DNA was eluted by adding 5 ml Buffer QF. The DNA was precipitated with 7 ml room-temperature isopropanol. The precipitated DNA was centrifuged for 30 mins at 12,000 rpm at 4ºC. The supernatant was poured off and then 5 ml 70% ethanol was added into the tube. The tube was centrifuged for 5 min at 12,000 rpm at 4ºC. The supernatant was poured off. The DNA was dried for 5 min in the air and then re-suspended in 1 ml $dH_2O$ or TE.

## Transfection of Intact Cerebral Arteries

A Rab11A sequence with a serine 177-to-alanine mutation (Rab11A S177A) was generated and sub-cloned into pcDNA3.1(+) (GenScript USA Inc., Piscataway, NJ). Empty pcDNA3.1 vectors, pcDNA3.1 encoding Rab11A, or pcDNA3.1 encoding Rab11A S177A were transfected into arteries using electroporation (CUY21Vivo-SQ electroporator; Bex), as previously described. (Narayanan, Bulley et al. 2013) Cerebral arteries were placed in an electroporation chamber containing $Ca^{2+}$- free PBS with either Rab11A WT, Rab11A S177A or empty vectors. Tandem-pulse electroporation was applied to cerebral arteries eight times in the chamber. The pulse voltage was set at 50 mV. On-time application was set for 50 ms. Arteries were then placed in serum-free DMEM-F12 media supplemented with 1% penicillin-streptomycin for 3 days before use.

## HEK293 Cell Culture and Transfection

Human Embryonic Kidney 293 cells (HEK293, ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Cellgro, Manassas, VA) and 1% penicillin-streptomycin under standard culture conditions (21% $O_2$-5% $CO_2$; 37°C). Cells were transiently transfected with pcDNA3.1(+) encoding full-length β1 (2 μg) or empty vector (2 μg) using Effectene transfection kit (Qiagen). HEK293 cells were cultured on sterile petri dishes in a 74% $N_2$/21% $O_2$/5% $CO_2$ incubator to achieve confluency. Cells were lysed after 48 hours post-transfection. Protein concentration was calculated as previously described. Cell lysates were run on 7.5% SDS polyacrylamide gels and blots were probed for β1 and actin.

## Immunoprecipitation

Protein lysate was harvested from cerebral arteries from six rats using ice-cold radio-immunoprecipitation buffer. Proteins were pulled down from arterial lysate using the Catch and Release version 2.0 immunoprecipitation kit (Millipore) per the manufacturer's instructions. Concentrated wash buffer was diluted into the 1× concentration with water for incubation and following washes. The spin columns, centrifuge tubes and capture tubes were labeled to be used. The snap-off bottom plug was removed and the spin column was inserted into a capture tube. The screw-on cap was removed and centrifuged at 5000 rpm for 15s to remove the resin buffer. The resin was washed twice with 400μl 1× wash buffer. The capture tube was emptied and the bottom end of the column was plugged with the snap-off bottom plug.

Samples were incubated with Rab11A primary antibody, 0.5ml capture resin and 10μl antibody affinity ligand in columns at 4°C overnight. The columns were centrifuged at 5000 rpm and the flow through discarded. The capture resin was washed with 1X wash buffer twice. Bound proteins were released using denaturing buffer and then boiled for 3 mins. Protein samples were analyzed using Western blotting with anti-rab11A (1:500, Cell Signaling). The blots were stripped using Restore Western Blot Stripping Buffer

(Thermo Fisher Scientific) for reprobing with anti-phosphoserine antibody (1:500, Millipore).

## Patch Clamp Electrophysiology

### Inside-out patches

Single BK channels or transient BK currents were recorded in isolated myocytes using the inside-out or perforated patch-clamp configurations, respectively. Currents were recorded by an Axopatch 200B amplifier and Clampex 10.3. For inside-out patch-clamp, myocytes were allowed to settle in a recording chamber before exposure to combinations of ET-1 (1 hour), BIM (1 hour) and SNP (10 min) prior to patch excision. Inside-out patches were pulled and agents were washed out so that channel activity was measured in the absence of these agents using a holding potential of -40mV or +80mV. For inside-out recordings, the pipette and bath solution both contained (in mmol/L): 130 KCl, 10 HEPES, 5 EGTA, 1.6 HEDTA, 1 $MgCl_2$, and 10 μmol/L free $Ca^{2+}$ (pH 7.2).

### Perforated whole cell patches

Transient BK currents were measured using the amphotericin B perforated patch configuration at a steady voltage of -40mV. For perforated patch-clamp, the bath solution contained (in mmol/L): 134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4) and the pipette solution contained (in mmol/L): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, and 0.05 EGTA (pH 7.2). Currents were filtered at 1 kHz, digitized at 5 kHz and analyzed using Clampfit 10.3 (MDS Analytical Technologies).

## Pressurized Artery Myography

Artery segments (1-2 mm length) were cannulated in a perfusion chamber (Living Systems Instrumentation, St. Albans, VT) and continuously perfused with 37 ºC physiological saline solution (PSS) which contained: 112 NaCl, 4.8 KCl, 24 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $KH_2PQ_4$ and 10 glucose, gassed with mixture of 21% $O_2$, 5% $CO_2$ and 74% $N_2$. The endothelium was denuded by introducing an air bubble into the lumen for ~1 minute followed by wash with PSS. The procedure was carefully performed in order to avoid any stretch or damage to the arteries. Artery segments without any small branches were chosen to be cannulated in the chamber. If there were any small branches, they were tighten with thread. The chamber containing artery segment was placed on the stage of a Nikon TS100-F microscope. Intravascular pressure was achieved by raising a water-filled reservoir which was connected to the cannulated arteries. The pressure was monitored using a pressure transducer. Arterial diameter was measured using a CCD camera attached to a Nikon TS100-F microscope. The diameter changes were measured

in real time by the edge-detection function of IonWizard (Ionoptix, Milton, MA). The chamber was constantly perfused with PSS, or PSS containing indicated agents. Myographic traces were analyzed using IonWizard software. Myogenic tone (%) was calculated as: $100 \times (1 - D_{active}/D_{passive})$, where $D_{active}$ is active arterial diameter and $D_{passive}$ is the diameter determined in $Ca^{2+}$-free PSS supplemented with 5 mmol/L EGTA.

## Reagents and Chemicals

Sodium nitroprusside was purchased from Sigma-Aldrich Company (St. Louis, MO). Endothelin-1 was also purchased from Sigma-Aldrich Company (St. Louis, MO). Bisindolylmaleimide was purchased from Cayman Chemical Company (Ann Arbor, MI).

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism v4.0 and Origin v6.0. Values are presented as mean ± SEM. Student's t-test with two tail p value was used to compare paired and unpaired data from two populations. ANOVA with Student-Newman-Keuls post hoc test was used for multiple group comparisons. The values were assumed to be sampled from a Gaussian distribution. $P < 0.05$ was considered significant. Power analysis was performed to verify that the sample size gave a value of >0.8 if P was >0.05.

# CHAPTER 4. RESULTS*

## ET-1 Inhibits NO-induced Surface Trafficking of B1 Subunits via PKC Activation

To measure the cellular distribution of BKα and β1 subunits in myocytes of intact arteries, we used biotinylation, a method previously validated to label surface, but not intracellular proteins. (Bannister, Adebiyi et al. 2009, Kidd, Bulley et al. 2016)

Our data indicated that BKα subunits were primarily present in the plasma membrane, whereas β1 was mostly intracellular in resting resistance-size cerebral arteries (**Figure 4-1A, D**). ET-1 alone did not change the surface abundance of either BKα or β1 subunits (**Figure 4-1B**). To further study ET-1-modulation of subunits, sodium nitroprusside (SNP), a NO donor, was first applied to stimulate surface trafficking of β1, as previously demonstrated.(Leo, Bannister et al. 2014) SNP increased surface β1 protein ~2.6-fold, but did not alter surface BKα protein (**Figures 4-1A, 4-2B**). ET-1 inhibited the SNP-induced increase in surface β1 protein (**Figures 4-1A, 4-2B**). Changes in surface β1 were associated with corresponding shifts in intracellular β1, suggesting β1 protein redistributed from an intracellular compartment to the surface (**Figure 4-2B, C**). SNP and ET-1 did not alter total β1 protein, indicating that β1 subunits were not degraded (**Figure 4-2B, C**). SNP and ET-1 did not alter BKα distribution or total protein (**Figures 4-1D, 4-2D**). These data suggest that ET-1 inhibits NO-induced surface trafficking of β1 subunits in arterial myocytes.

Many vasoconstrictors, including ET-1, stimulate $G_{q/11}$ in myocytes, leading to an increase diacylglycerol, which activates protein kinase C (PKC).(Ivey, Osman et al. 2008, Wynne, Chiao et al. 2009) To examine mechanisms by which ET-1 decreased β1 subunit trafficking, bisindolylmaleimide (BIM, 1h), a PKC inhibitor, was used. BIM abolished the ET-1-induced inhibition of β1 subunit surface trafficking, essentially restoring SNP-induced stimulation of surface β1 protein (**Figure 4-1A, D**). In contrast, BIM did not alter surface β1 protein when applied alone (**Figure 4-1C**). BIM also did not change surface BKα when applied alone or together with ET-1 (**Figure 4-1C**). These data suggest that ET-1 activates PKC, which inhibits surface trafficking of β1 subunits in arterial myocytes.

## β1 Antibody Identifies HEK Cell Expressing β1 Subunits

In order to test β1 antibody identified β1 protein, HEK293 cells were transfected with pcDNA3.1-empty vectors or pcDNA3.1-BK β1 vectors and then cultured for three days. The total protein lysate was obtained from two groups of cultured HEK293 cells

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* Portions of this chapter adapted with permission. Zhai, X., M. D. Leo and J. H. Jaggar (2017). "Endothelin-1 Stimulates Vasoconstriction Through Rab11A Serine 177 Phosphorylation." Circ Res **121**(6): 650-661.

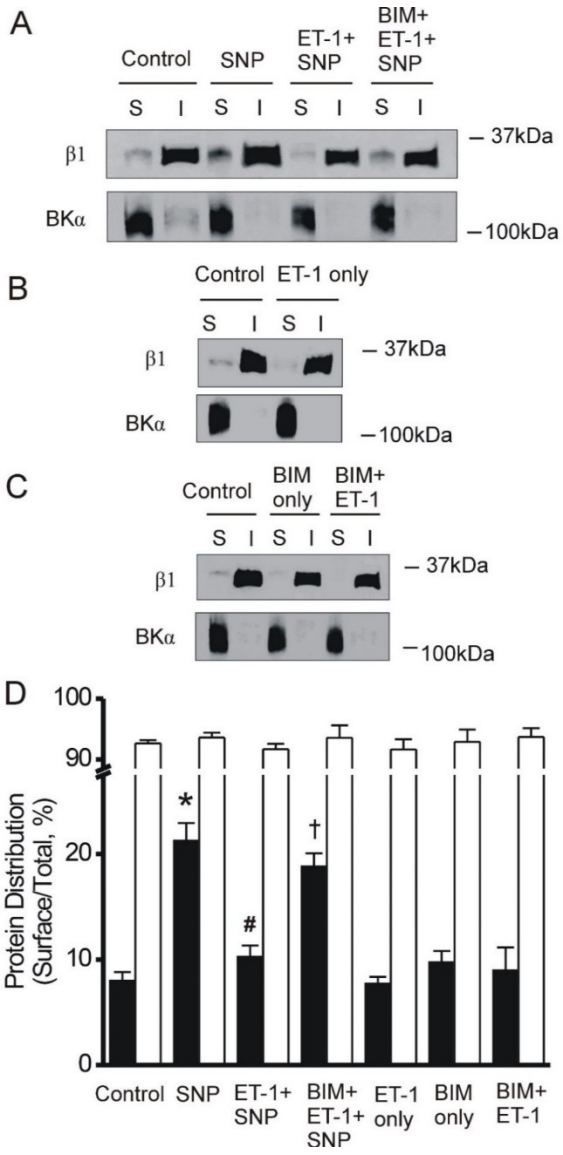


**Figure 4-1. ET-1 decreases NO-induced surface trafficking of β1 subunits via PKC activation.**

*Notes:* (A) Representative Western Blots indicating surface (S) and intracellular (I) BKα and β1 subunits. Rat cerebral arteries were exposed to: nothing (control), SNP (10 μmol/L, 10 min), SNP (10 μmol/L, 10 min) +ET-1 (10 nmol/L, 1h) or BIM (20 μmol/L, 1h)+ SNP (10 μmol/L, 10 min) + ET-1 (10 nmol/L, 1h). (B) Representative Western Blots indicating surface (S) and intracellular (I) BKα and β1 subunits. Rat cerebral arteries were exposed to: nothing (control) and ET-1 (10 nmol/L, 1h) only. (C) Representative Western Blots indicating surface (S) and intracellular (I) BKα and β1 subunits. Rat cerebral arteries were exposed to: nothing (control), BIM (20 μmol/L, 1h) only and BIM (20 μmol/L, 1h) +ET-1 (10 nmol/L, 1h). (D) Mean data illustrating BKα and β1 subunit protein distribution (= Surface protein / Total protein) (n=6 for each). *$p$<0.05 vs. control, # $p$<0.05 vs SNP, † $p$<0.05 vs. ET-1+SNP.

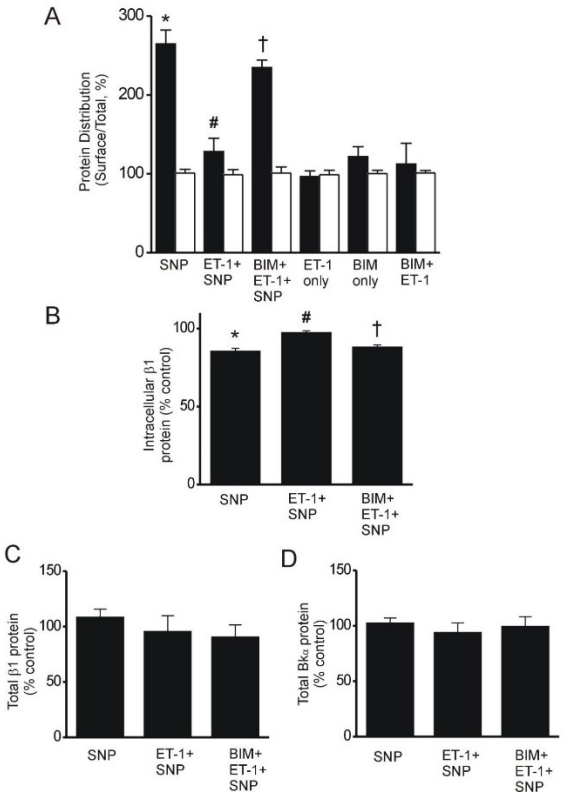


**Figure 4-2. SNP, ET-1, BIM, or combination of these agents do not change total protein of BKα and β1 subunits after surface biotinylation.**

*Notes:* (A) Mean surface protein data from biotinylation experiments (n=6 for each). **p*<0.05 vs. control, # *p*<0.05 vs SNP, † *p*<0.05 vs. ET-1+SNP. (B) Mean data for intracellular β1 and BKα protein (n=6 for each). *p<0.05 vs. control, # p<0.05 vs. SNP, † p<0.05 vs. ET-1+SNP. (C) Mean data for total β1 protein compared to control (n=6 for each). (D) Mean data for total BKα protein compared to control (n=6 for each).

and run on Western blotting. The β1 antibody did not identify a protein in lysate from mock-transfected HEK293 cells, but identified a ~34 kDa protein in cells expressing recombinant β1, which was of a similar molecular mass to that of arterial β1 protein (**Figure 4-3**).

These experiments provide additional support for previous evidence that the biotinylation procedure does not label intracellular proteins and that the β1 subunit antibodies detect β1 protein.

## ET-1 Inhibits Depolarization-induced Surface Trafficking of B1 Subunits in Arterial Myocytes

Experiments were performed to investigate whether ET-1 inhibition of β1 subunit surface trafficking is specific to NO or a more general inhibitory mechanism. Membrane depolarization also stimulates an increase in surface β1 subunits, leading to BK channel activation.(Leo, Zhai et al. 2017) Membrane depolarization (30 mmol/L $K^+$) increased surface β1 protein 3.70-fold, but did not alter surface BKα in cerebral arteries (**Figure 4-4A, B**). ET-1 inhibited the depolarization-induced increase in surface β1 protein (**Figures 4-4A, B**). BIM reduced the ET-1-induced inhibition of β1 subunit surface trafficking by ~ 60 % (**Figure 4-4A, B**). These data suggest that ET-1 stimulation of PKC inhibits both NO- and depolarization-induced surface trafficking of β1 subunits in arterial myocytes.

Control experiments were performed to examine both the arterial biotinylation procedure and the β1 subunit antibodies. Reprobing blots from arterial biotinylation experiments indicated that cytochrome C, a mitochondrial protein, was detected only in the intracellular (i.e. non-biotinylated) fraction (**Figure 4-4A**). There were no significant difference of cytochrome C expression among these group, indicating that the total among of protein was similar.

## ET-1 Decreases NO-induced N-FRET Between B1 and Bkα Subunits via PKC Activation

Förster resonance energy transfer (FRET) imaging followed by N-FRET analysis was used to measure BKα and β1 subunit colocalization and spatial proximity in isolated cerebral artery myocytes. In control, the majority of BKα subunits were localized at the cell periphery, whereas β1 subunits were primarily intracellular. N-FRET between BKα and β1 subunit-bound secondary antibodies in control was low, as previously demonstrated (Leo, Bannister et al. 2014) (**Figure 4-5A, B**).

After myocytes were exposed to SNP (10μM, 10mins), SNP increased N-FRET ~2.8-fold with FRET located at the cell surface (**Figure 4-5A, B**). It indicated that SNP induced surface trafficking β1 subunits, which associated with BKα subunits so that N-FRET between two subunits were increased.

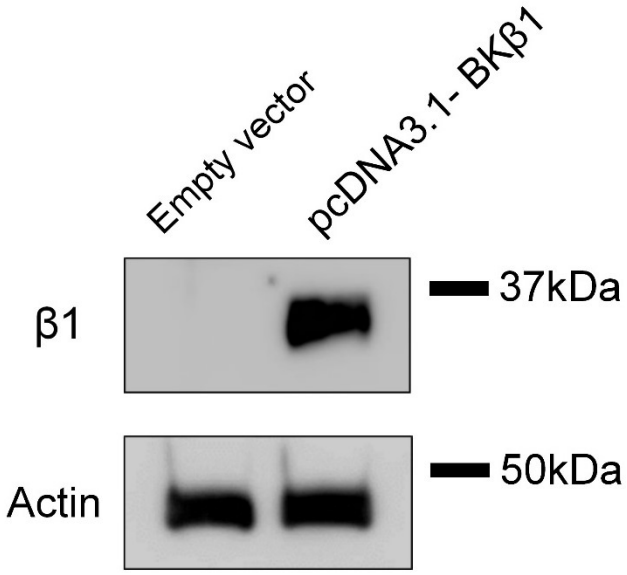


**Figure 4-3. Anti-β1 antibody only detects HEK293 cells transfected with BK β1 vectors.**

*Note:* Representative western blotting indicating β1 protein and actin. HEK293 cells were transfected with empty vectors (left lane) or BK β1 vectors (right lane) and total protein lysates were run on western blotting and probed with anti-β1 protein antibody and anti-actin antibody.

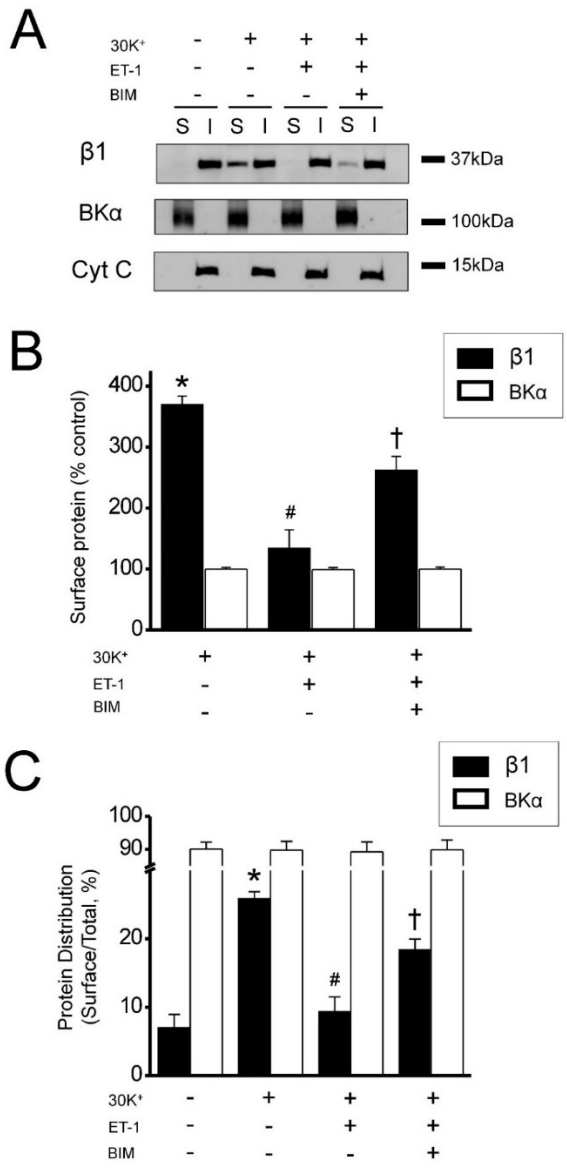


**Figure 4-4. ET-1 decreases depolarization-induced surface trafficking of β1 subunits via PKC activation.**

*Notes:* (A) Representative Western Blots indicating surface (S) and intracellular (I) BKα, β1 and cytochrome C (cyt C). Arteries were exposed to: 6 mmol/L $K^+$ (control), 30 mmol/L $K^+$ (10 min), ET-1 (10 nmol/L, 1h), and BIM (20 µmol/L, 1h) alone or in combinations indicated. (B) Mean surface protein data from biotinylation experiments (n=5 for each). **p*<0.05 vs. control, # *p*<0.05 vs 30 mmol/L $K^+$, † *p*<0.05 vs. 30 mmol/L $K^+$ + ET-1. (C) Mean data illustrating BKα and β1 subunit protein distribution (n=6 for each). **p*<0.05 vs. control, # *p*<0.05 vs 30 mmol/L $K^+$, † *p*<0.05 vs. 30 mmol/L $K^+$ + ET-1.

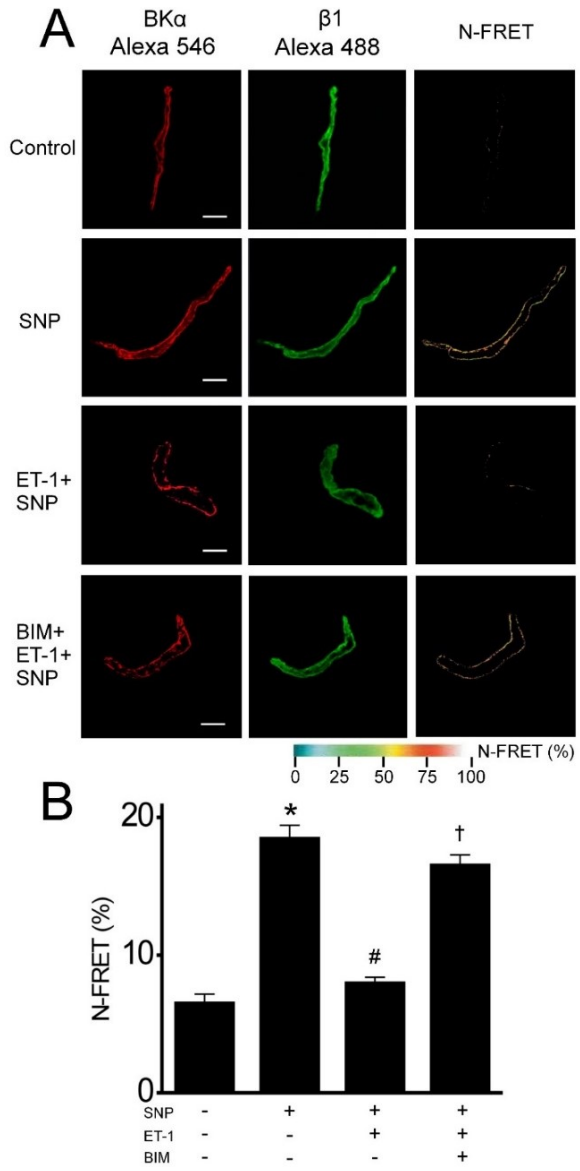


**Figure 4-5. ET-1 decreases NO-induced N-FRET between β1 and BKα subunits via PKC activation.**

*Note:* (A) Representative immunofluorescence and immuno-FRET images of BKα and β1 in arterial myocytes. Scale bars, 10μm. SNP (10 μmol/L, 10 min), ET-1 (10 nmol/L, 1h), BIM (20 μmol/L, 1h) were applied alone or in combinations indicated. (B) Mean data for BKα and β1 immuno-FRET (%). (n=10 for each) *$p$<0.05 vs. control, # $p$<0.05 vs SNP, † $p$<0.05 vs. ET-1+SNP.

To examine ET-1 inhibited the co-assembly between β1 and BKα subunits, myocytes were first exposed to ET-1 (10nM, 1h). SNP (10uM) was added at last 10 mins. Endothelin-1 reduced the N-FRET from 19% in the SNP group to about 6.8%, which means that ET-1 inhibited the ability of SNP to increase N-FRET. (**Figure 4-5A, B**)

In contrast, BIM restored N-FRET to about 17%, which demonstrated that the inhibitory effect was blocked by BIM (**Figure 4-5A, B**). These data suggest that ET-1 activates PKC which inhibits β1 subunit surface trafficking, thereby blocking plasma membrane co-assembly of β1 with BKα subunits in arterial myocytes.

### ET-1 Inhibits NO-induced Po of BK Channels via PKC Pathway

To investigate BK channel regulation by ET-1, patch-clamp electrophysiology was performed. Isolated arterial myocytes were exposed to nothing (control), SNP, ET-1+SNP or SNP+ET-1+BIM. Inside-out patches were pulled from myocytes and agents washed out with bath solution to measure channel properties under the same experimental conditions and to remove any direct effects of pharmacological modulators on activity. BK channel activity was measured with 10 μM free intracellular $Ca^{2+}$ concentration at either -40mV, a physiological voltage, or +80mV to stimulate maximal activation.

In excised patches from control myocytes, mean BK channel open probability was ~0.20 (**Figure 4-6A, B**). Myocytes were exposed to SNP for 10min. After inside out patches were pulled from myocytes, SNP were washed out. SNP treatment of myocytes increased BK channel mean $P_o$ to ~0.47, or ~2.35-fold (**Figure 4-6A, B**). Then the myocytes were treated with ET-1 for 1h before last 10mins SNP treatment. ET-1 reduced SNP-induced BK channel activation to ~40.0% of that stimulated by SNP alone (**Figure 4-6A, B**). BIM blocked the ET-1-induced reduction in BK channel $P_o$ (**Figure 4-6A, B**).

SNP, ET-1+SNP or BIM+ET-1+SNP did not alter BK channel maximal $P_o$ or the mean number of channels in patches (1.3±0.2, 1.5±0.3, 1.2±0.2, 1.3±0.3 channels in control, SNP, ET-1+SNP, BIM+ET-1+SNP, respectively; $p>0.05$) (**Figure 4-6A, B**). When combined with the biochemical data here, these data indicate that ET-1 activates PKC, which inhibits surface trafficking of β1 subunits, leading to a decrease in BK channel $P_o$.

### NO Stimulates Active Rab11 and ET-1 Inhibits Active Rab11

NO stimulates Rab11A-positive recycling endosomes to deliver β1 subunits to the plasma membrane.(Leo, Bannister et al. 2014) Cerebral arteries were exposed to nothing (control), SNP (10 μmol/L) or SNP (10 μmol/L) + ET-1 (10 nmol/L). Total protein lysates were obtained and run on Western blotting. To examine the molecular mechanism by which ET-1 stimulated-PKC inhibits β1 trafficking, we measured Rab11A activation in cerebral arteries using Western blotting and a monoclonal antibody that recognizes

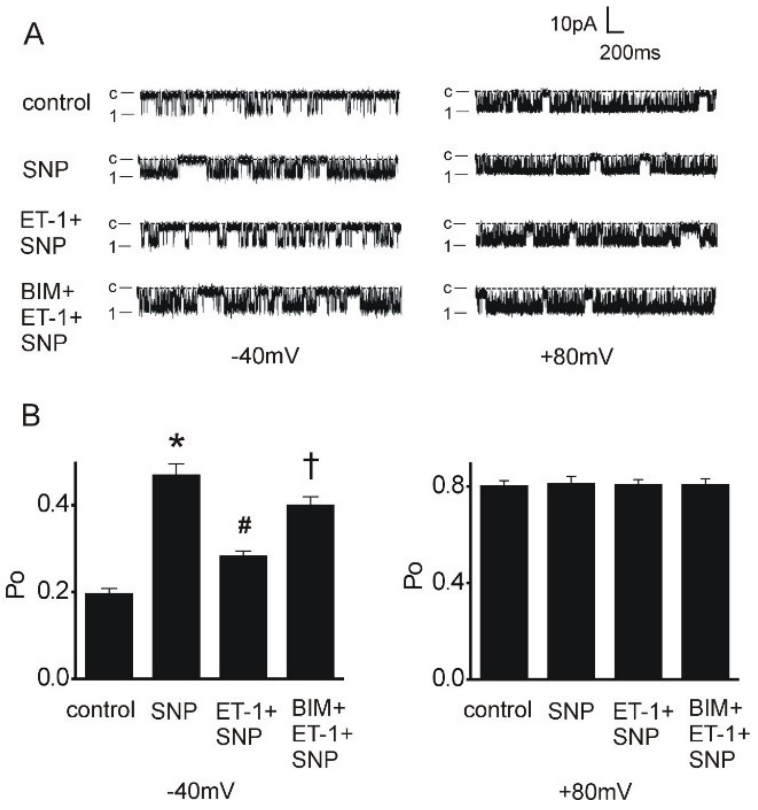


**Figure 4-6. ET-1 inhibits BK channels via a PKC-dependent mechanism.**

*Notes:* (A) Fresh, isolated arterial myocytes were exposed to nothing (control), SNP (10 μmol/L), ET-1 (10 nmol/L) + SNP (10 μmol/L), or BIM (20 μmol/L) + ET-1 (10 nmol/L) + SNP (10 μmol/L) after which inside-out patches were pulled and BK channel activity recorded in the absence of the agents. Representative traces illustrate BK channel activity recorded in 10μM free $Ca^{2+}$ in the same patches at -40mV or +80mV. (B) Mean BK channel $P_o$ values obtained from fresh-isolated myocytes at -40mV and +80mV. (n=6 for each) **p*<0.05 vs. control, # *p*<0.05 vs SNP, † *p*<0.05 vs. ET-1+SNP. & *p*<0.05 vs. ET-1+SNP in mock group.

Rab11-GTP, but not Rab11-GDP. SNP increased active Rab11 ~2.62 fold (**Figure 4-7A, B**). ET-1 reduced active Rab11 to ~28.4% of that in SNP alone (**Figure 4-7A, B**). These data suggest that NO activates Rab11 and ET-1 inhibits Rab11 in arterial myocytes.

## ET-1 Inhibits Rab11A Through Phosphorylation at Serine 177

### Bioinformatics study showed that Serine 177 in Rab11A has highest probability of phosphorylation by PKC

The mechanism by which PKC reduces Rab11A activity may occur either directly or indirectly through other signaling intermediates. We tested the hypothesis that PKC directly phosphorylates Rab11A to reduce activity. Analysis of the Rab11A amino acid sequence (NetPhosK 1.0) revealed five potential PKC phosphorylation sites, of which Ser177 had the highest probability (**Table 4-1**). We constructed a Rab11A S177A mutant to study physiological functions of serine 177 in β1 subunit trafficking. Basically, the codon which stands for Ser177 was mutated in to alanine by using site directed mutagenesis. The wild type rat's Rab11A cDNA or Rab11A S177A cDNA was sub-cloned into pcDNA3.1+. Our strategy is that we simply mutated one base pair so that it can conserve the function of protein as much as possible. We mutated TCT (serine) into GCT (alanine). By using site directed mutagenesis, the basic idea is that the forward and backward primers are designed that containing the mutant base pair. During performing thermal cycling, mutant primers can also anneal to the wild type Rab11A plasmids and then synthesize Rab11A S177A. Then we transformed the plasmids into competent cells to generate more plasmids.

### Rab11A WT or Rab11A S177A similarly increases the total protein of Rab11A

To investigate the hypothesis that ET-1 phosphorylates Rab11A S177, cerebral arteries were transfected with vectors encoding either wild-type Rab11A (Rab11A WT) or Rab11A S177A. Cerebral arteries were cultured and then homogenized to get protein lysate. The total protein lysate was run on Western Blotting and total Rab11A protein was compared among three groups. Vectors encoding wild-type Rab11A or Rab11A S177A similarly increased total Rab11A protein, to ~190% of mock controls **(Figure 4-8A, B**). Rab11A WT or Rab11A S177A did not alter levels of BKα, β1 or actin proteins (**Figure 4-8A, B).**

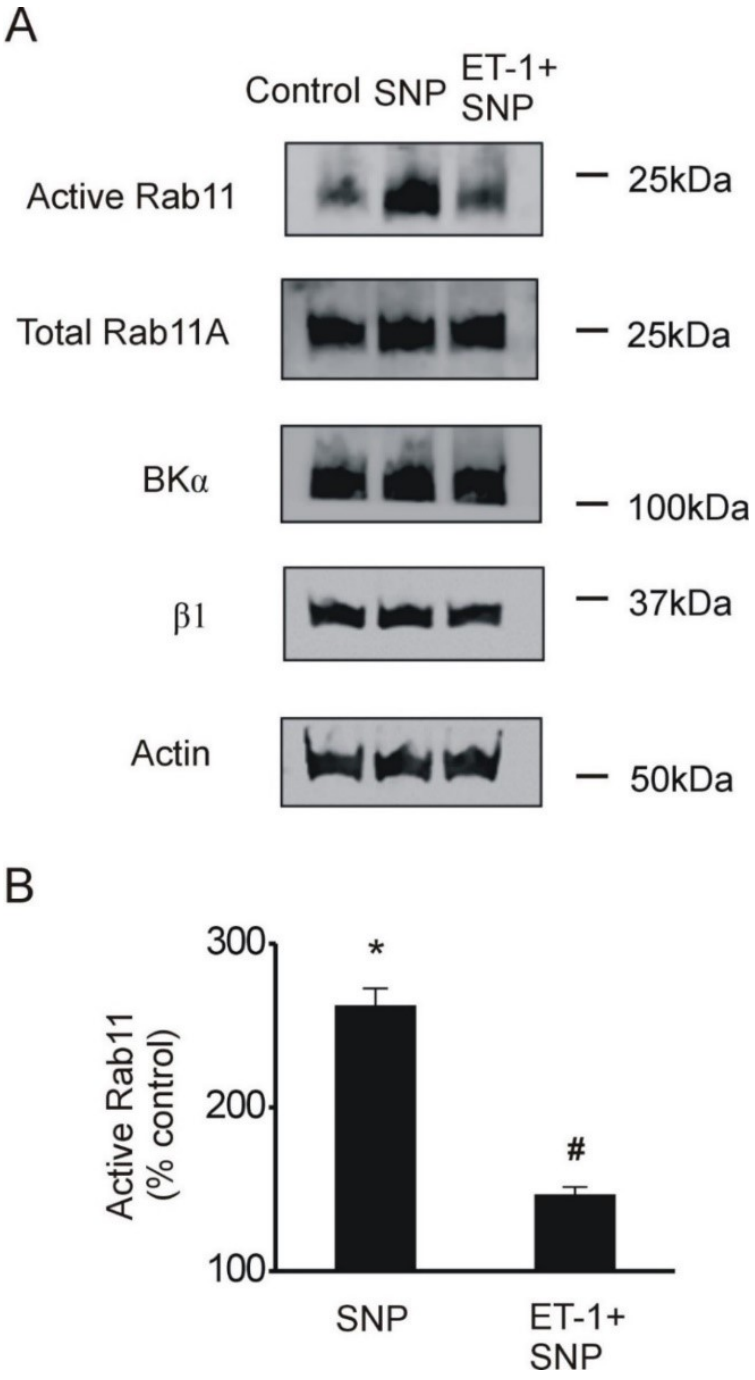


**Figure 4-7. NO stimulates active Rab11, ET-1 inhibits active Rab11.**

*Notes:* (A) Representative Western Blots of active Rab11 and total Rab11A, BKα, β1 and actin in control arteries and arteries treated with SNP (10 μmol/L, 10 min) or SNP + ET-1 (10 nmol/L, 1h). (B) Mean data of active Rab11 in SNP and SNP+ET-1 group compared to control (n=6). *$p$<0.05 vs. control, # $p$<0.05 vs SNP.

**Table 4-1. Potential PKC phosphorylation sites in Rab11A, of which S177 has the highest probability.**

| Site | Kinase | Score |
|---|---|---|
| T-59 | PKC | 0.70 |
| S-78 | PKC | 0.53 |
| T-170 | PKC | 0.57 |
| S-177 | PKC | 0.75 |
| T-203 | PKC | 0.65 |

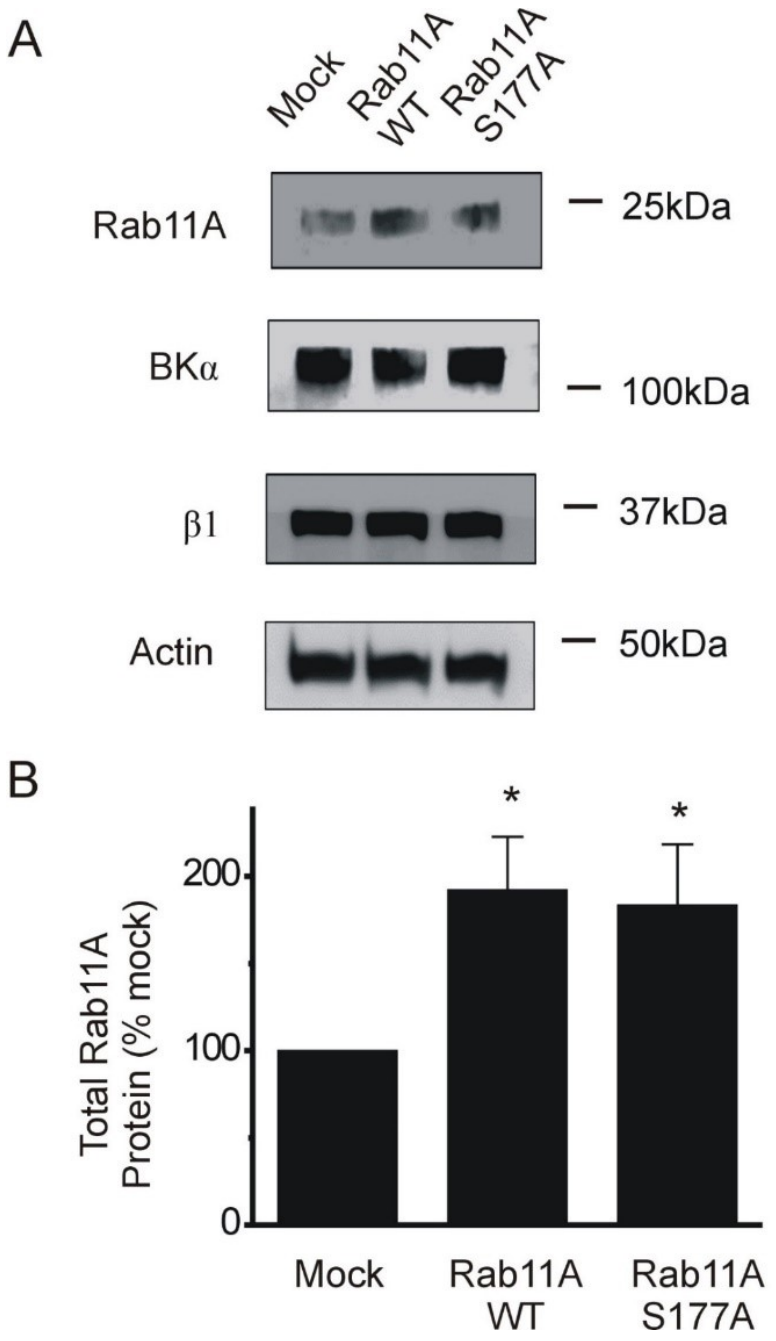


**Figure 4-8. Rab11A WT or Rab11A S177A similarly increases total Rab11A protein.**

*Notes:* (A) Representative Western Blots probed for total Rab11A, BKα, β1 or actin protein in arteries transfected with either an empty vector (mock) or a vector encoding Rab11A WT or Rab11A S177A. (B) Mean data of Rab11A total protein in Rab11A WT and Rab11A S177A group compared to mock (n=6). *$p$ <0.05 vs. mock group.

## Anti-Phosphoserine Is Increased in ET-1-treated Cerebral Arteries Expressing Rab11A WT but Not Rab11A S177A

Cerebral arteries were transfected with Rab11A or Rab11A S177A and cultured for three days. Arteries expressing either wild-type Rab11A or Rab11A S177A were exposed to ET-1 or nothing (control). Then, Rab11A protein was pulled down from total protein lysate and run on Western blots.

ET-1 did not alter the amount of total Rab11A in arteries expressing either wild-type Rab11A or Rab11A S177A (**Figure 4-9A, B, C**). Reprobing blots with an anti-phosphoserine antibody indicated that in the absence of ET-1, basal Rab11A phosphorylation was similar in arteries expressing either Rab11A WT or Rab11A S177A (**Figure 4-9A, B, C**). ET-1 increased Rab11A phosphorylation ~227 % in arteries expressing wild-type Rab11A. In contrast, ET-1 increased Rab11A phosphorylation only ~138 % in arteries expressing Rab11A S177 or ~60 % of that in ET-1-treated arteries expressing Rab11A WT (**Figure 4-9A, B, C**). These data indicate that ET-1 phosphorylates serine 177 in Rab11A, leading to Rab11A inhibition in arterial myocytes.

## ET-1 Inhibits β1 Subunit Surface Trafficking Through Rab11A Serine 177

Experiments were performed to investigate the regulation of β1 trafficking by Rab11A S177 in arterial myocytes. Cerebral arteries were transfected with empty vectors or Rab11A S177A and then cultured for three days. After surface biotinylation, surface and intracellular protein under each condition were run on Western blotting and then probed with BKα and β1 antibodies.

The data showed that SNP increased surface β1 protein similarly in mock and Rab11A S177A groups (**Figure 4-10A, B**). In the mock group, ET-1 reversed the SNP-induced increase in β1 subunit surface protein (**Figure 4-10A, B**). Rab11A S177A expression blocked the ability of ET-1 to inhibit β1 subunit surface expression (**Figure 4-10A, B**).

## Rab11A S177A Restores Co-assembly Between BKα and β1 Subunits in the Presence of ET-1

Cerebral arteries were transfected with empty vectors (control) or Rab11A S177A and then cultured from three days. Then smooth muscle cells were isolated from these cerebral arteries. Both group of myocytes were exposed to ET-1 (10 nmol/L, 1h) and then SNP (10 μmol/L, 10 min). After treatment, myocytes were fixed and probed with primary antibodies and then secondary antibodies.

Confocal imaging of isolated cerebral artery myocytes illustrated that ET-1 decreased SNP-induced N-FRET signals in myocytes transfected with empty vectors, which was similar as fresh isolated myocytes. In contrast, Rab11A S177A expression prevented the ET-1-induced reduction in N-FRET between BKα and β1-subunit bound

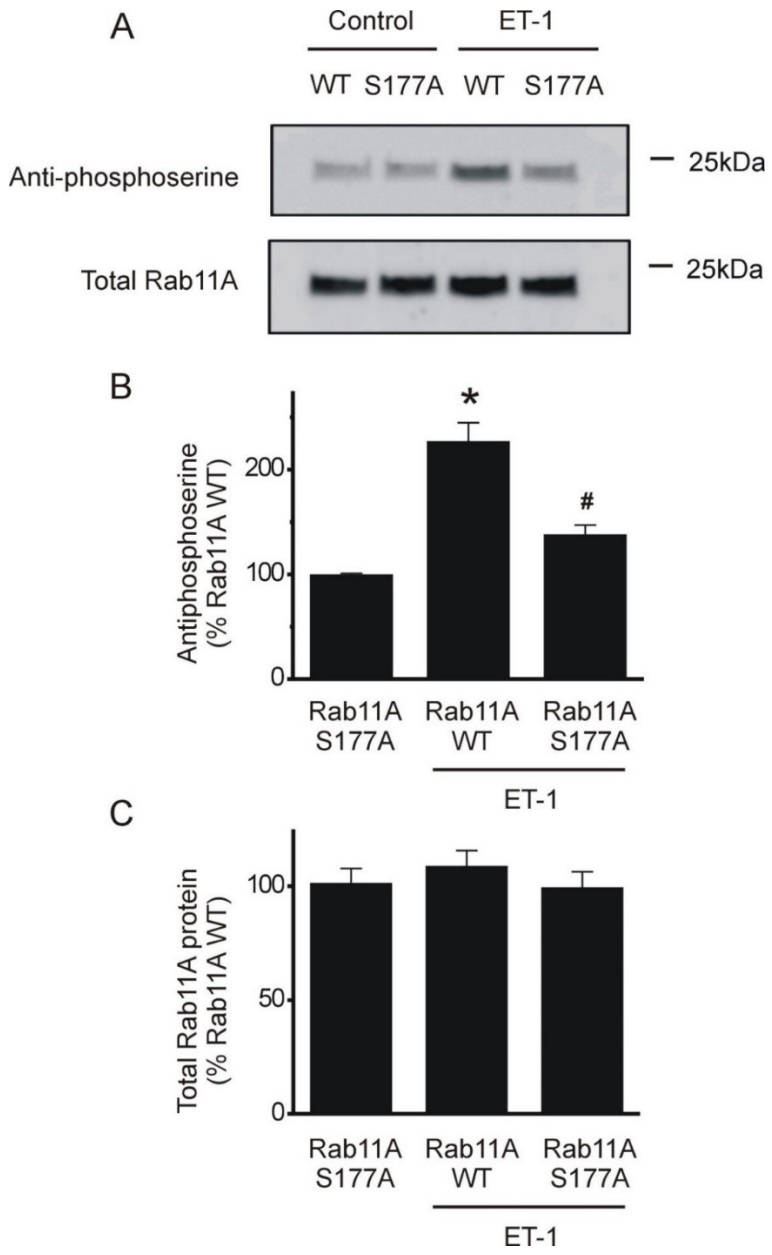


**Figure 4-9. Anti-phosphoserine is increased in ET-1-treated cerebral arteries expressing Rab11A WT but not Rab11A S177A.**

*Notes:* (A) Representative Western Blots probed with anti-phosphoserine or Rab11A antibodies after immunoprecipitation of Rab11A. (B) Mean data of anti-phosphoserine band intensity compared to untreated Rab11A WT (n=6). **p* <0.05 vs. Rab11A WT without ET-1 treatment, # *p* <0.05 vs. Rab11A WT with ET-1 treatment. (C) ET-1 did not change total Rab11A protein in arteries transfected with either Rab11A WT or Rab11A S177A. Mean data of total Rab11A protein expression in ET-1-treated cerebral arteries transfected with Rab11A WT or Rab11A S177A compared to non-ET-1-treated Rab11A WT groups (n=6).

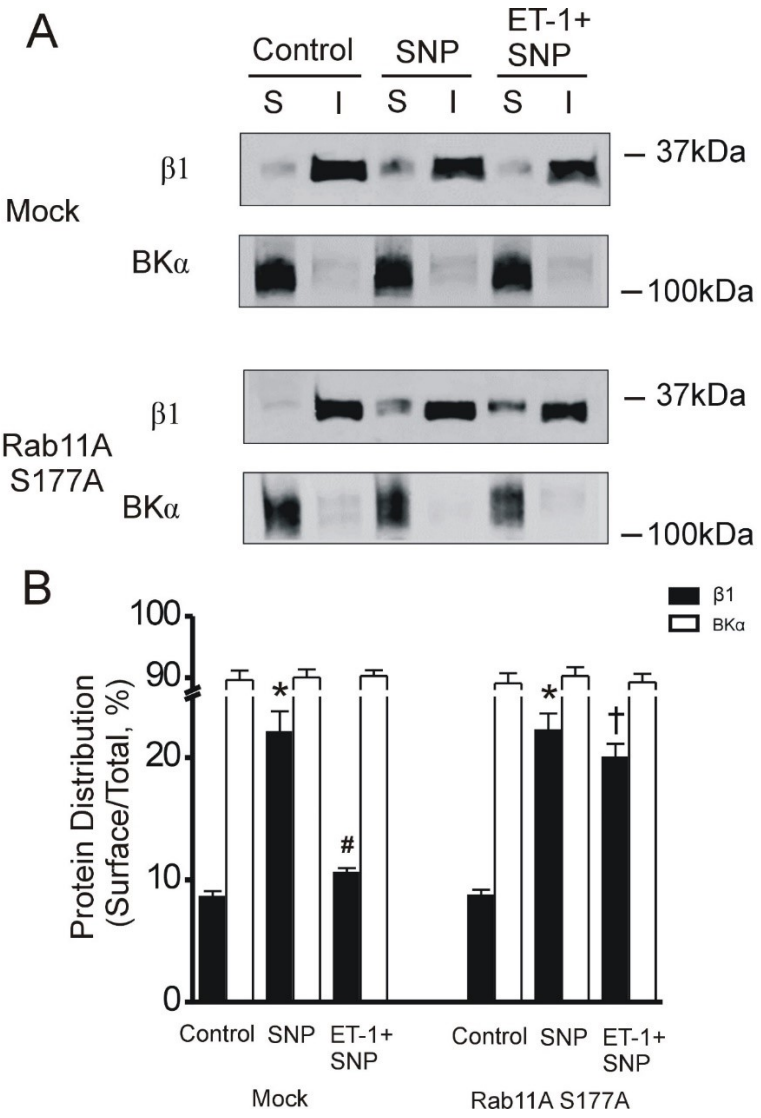


**Figure 4-10. ET-1 inhibits β1 subunit surface trafficking through Rab11A serine 177.**

*Notes:* (A) Representative Western blots illustrating surface (S) and intracellular (I) β1 and BKα protein in arteries transfected with an empty vector or a vector encoding Rab11A S177A in control, SNP (10 μmol/L, 10 min) or SNP (10 μmol/L, 10 min) + ET-1 (10 nmol/L, 1h), as indicated. (B) Mean data of protein distribution (n=6 for each). *$p$<0.05 vs. control (no agents), # $p$<0.05 vs. SNP in the mock group, † $p$<0.05 vs. ET-1+SNP in the mock group.

secondary antibodies (**Figure 4-11C, D**). Taken together, these data suggest that ET-1-induced PKC acts through Ser177 on Rab11A, leading to a reduction in Rab11A activity and inhibition of β1 subunit surface trafficking in arterial myocytes.

### ET-1 Inhibits BK Channels Through PKC-mediated Phosphorylation of Rab11A

Given that Rab11A S177A expression attenuated ET-1-inhibition of β1 surface expression, we measured BK channel regulation by this mutant construct in arterial myocytes. Cerebral arteries were transfected with empty vectors (control) or Rab11A S177A and then cultured from three days. Then smooth muscle cells were isolated from these cerebral arteries. Both group of myocytes were exposed to ET-1 (10 nmol/L, 1h) and then SNP (10 μmol/L, 10 min). Cell patches were pulled and then the chamber was washed with intracellular solution. The single channel activity of BK channel was measured at -40mV and +80mV.

The mean $P_o$ of BK channels in mock-transfected cells treated with ET-1+SNP was similar to that non-transfected, fresh-isolated cells in the same experimental condition (**Figure 4-12A, B**). Rab11A S177A expression blocked the ability of ET-1 to inhibit BK channels (**Figure 4-12A, B**). Rab11A S177A did not alter BK channel maximal $P_o$ (**Figure 4-12A, B**) or the number of channels in patches (2.2±0.1 and 2.3±0.2 channels in mock and Rab11A S177A, respectively; $p>0.05$). These data suggest that ET-1 inhibits BK channels through Rab11A S177 in arterial myocytes.

### ET-1 Inhibits Transient BK Currents Through Rab11A S177

In arterial myocytes, BK channels are activated by $Ca^{2+}$ sparks. (Jaggar, Porter et al. 2000) A single $Ca^{2+}$ spark activates multiple nearby BK channels, leading to a transient BK current. To examine the functional relevance of Rab11A S177 on $Ca^{2+}$ spark-induced BK channel activation, transient BK currents were recorded in isolated arterial myocytes at a steady holding potential of -40 mV. Mean area under the curve (Inoue, Yanagisawa et al.) was calculated for each transient BK current (**Figure 4-13A**).

SNP similarly increased mean transient BK current AUC in mock-transfected cells and in cells expressing Rab11A S177A, by ~2.46- and ~2.42-fold, respectively (**Figure 4-13C**). In mock-transfected cells, ET-1 attenuated the SNP-induced increase in mean transient BK current AUC (**Figure 4-13A, C**). In contrast, Rab11A S177A expression inhibited the ability of ET-1 to reduce mean transient BK current AUC (**Figure 4-13B, C**). Rab11A S177A did not alter frequency modulation of transient BK currents by SNP or ET-1 (**Figure 4-13D**). These data suggest that ET-1 reduces transient BK current AUC through Rab11A S177. In contrast, Rab11A S177 does not modulate transient BK current frequency in arterial myocytes.

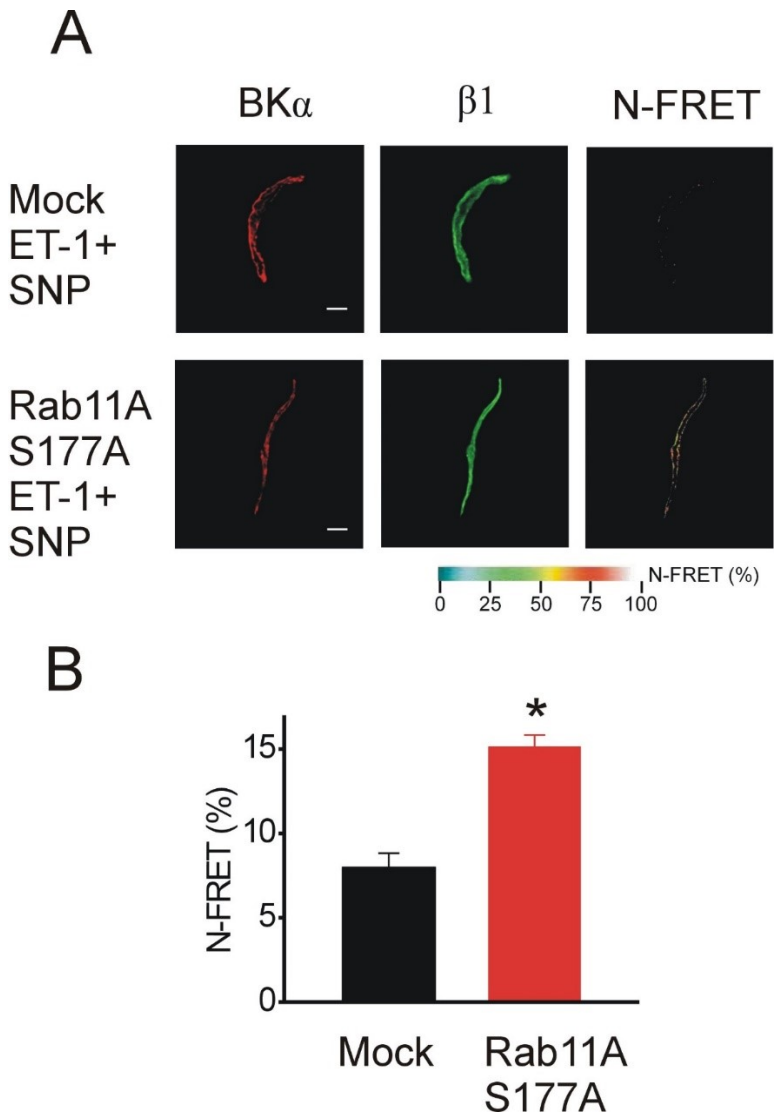


**Figure 4-11. Rab11A S177A restores co-assembly between BKα and β1 subunits in the present of ET-1.**

*Notes:* (A) Representative immunofluorescence and immuno-FRET images for BKα and β1 protein in arterial myocytes treated with SNP (10 μmol/L, 10 min) + ET-1 (10 nmol/L, 1h). Scale bars, 10 μm. (B) Mean N-FRET (%) data between BKα and β1 protein of mock group (black bar) and Rab11A S177A group (red bar). (n=10 for each) **p* <0.05 vs. the mock group.

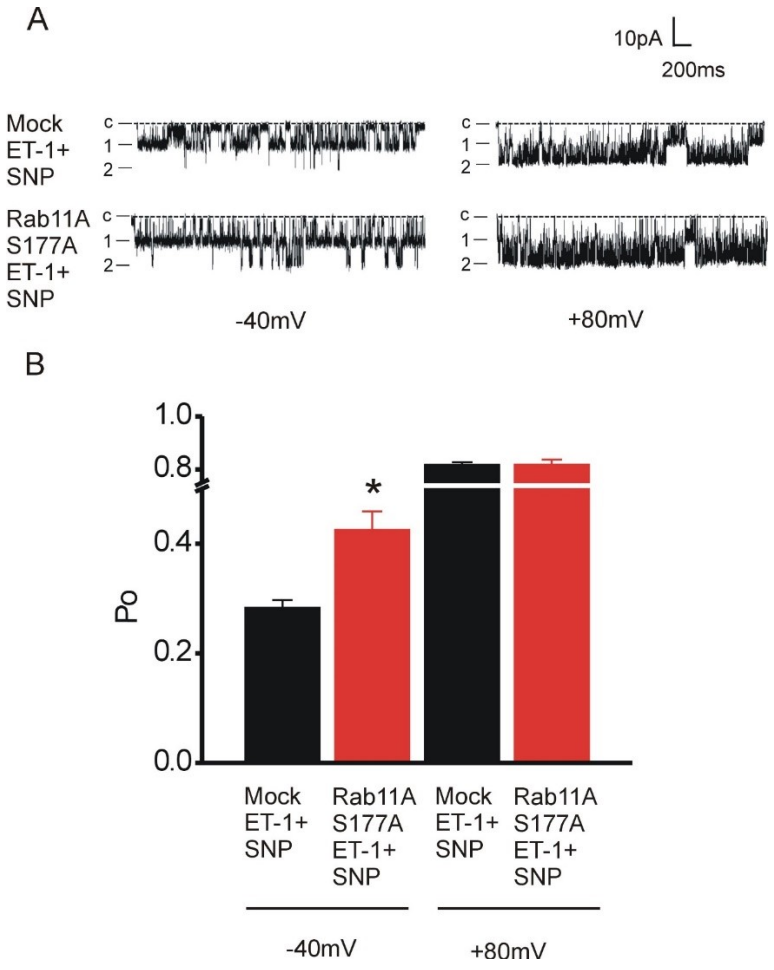


**Figure 4-12. ET-1 inhibits BK channels via Rab11A S177.**

*Notes:* (A) Representative BK channel recordings in inside-out patches pulled from empty vector-transfected (mock) and Rab11A S177A vector-transfected myocytes after exposure to ET-1 (10 nmol/L) + SNP (10 µmol/L) at -40mV and +80mV. (B) Mean BK channel $P_o$ values obtained from mock-transfected (black bars) and Rab11A S177A vector-transfected (red bars) myocytes at -40mV and +80mV. (n=6 for each) *$p$<0.05 vs. control.

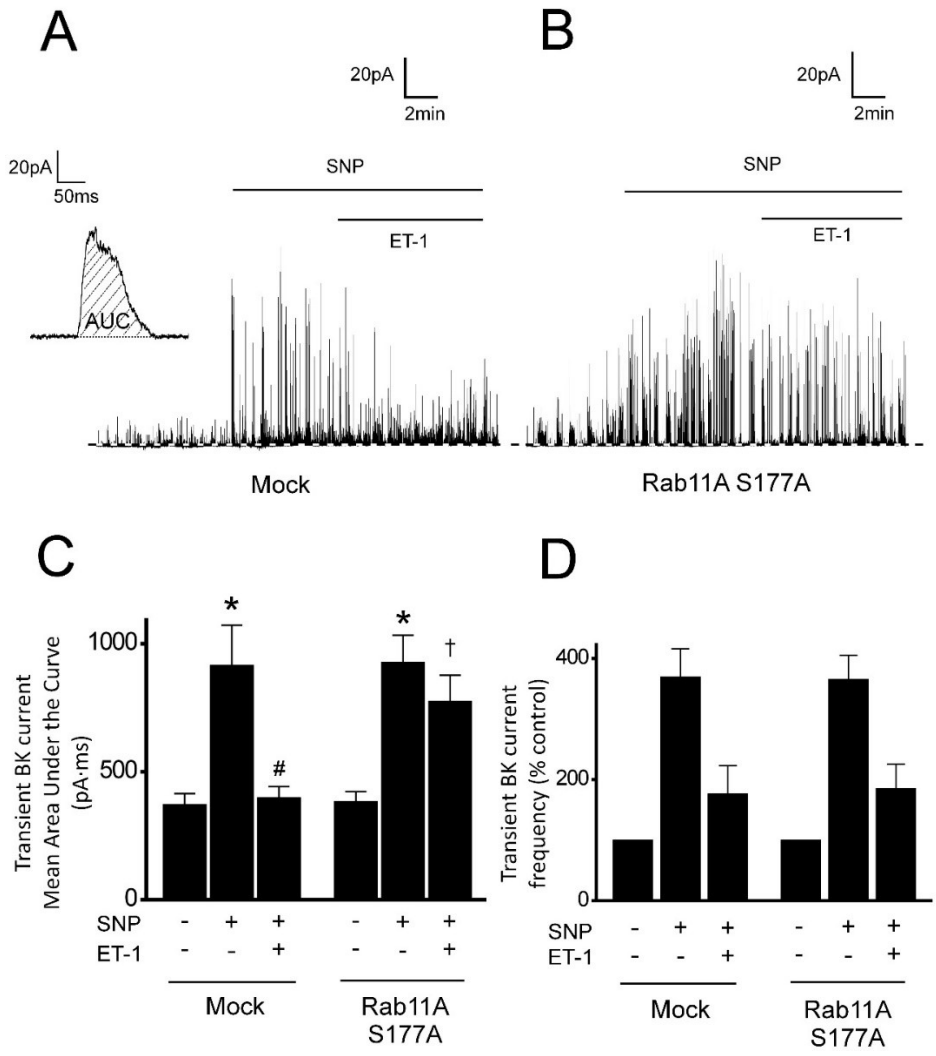


**Figure 4-13. Rab11A S177A attenuates ET-1-inhibition of transient BK currents.**

*Notes:* Representative recordings of transient BK currents measured in arterial myocytes transfected with an empty vector (mock, A) or a vector encoding Rab11A S177A (B) exposed to SNP (10 µmol/L) and ET-1 (10 nmol/L) at -40mV. The inset (A) indicates area under the curve of one transient outward current. (C) Mean data for BK current area under the curve (pA·ms, n=6 for each). * $p<0.05$ vs. control in mock group, # $p<0.05$ vs. SNP in mock group, † $p<0.05$ vs. ET-1+SNP in mock group. (D) Mean data for transient BK current frequency (n=6 for each).

## ET-1 Stimulates Vasoconstriction Through Rab11A S177 in Pressurized Arteries

The functional significance of Rab11A-mediated β1 subunit trafficking was studied in pressurized, myogenic (60 mmHg) arteries. After the arteries developed tone at 60 mmHg, arteries were applied to $60K^+$ PSS to induce vasoconstriction. The $60K^+$ response was then washed away by normal PSS. The arteries were then exposed to 10μM or 1μm SNP, which was dissolved in PSS. SNP similarly dilated mock-transfected and Rab11A S177A-transfected cerebral arteries (**Figure 4-14A, C**).

ET-1 applied in the continued presence of SNP stimulated vasoconstriction in both mock-transfected and Rab11A S177A-transfected arteries. However, in the presence of SNP, ET-1-induced vasoconstriction in Rab11A S177A arteries was ~42.5% less than in mock arteries under the same condition (**Figure 4-14A, C**).

Rab11A S177A expression also reduced vasoconstriction to ET-1 (1-10 nmol/L) alone to between 41.2 and 57.5 % of controls (**Figure 4-14A, C**). In contrast, Rab11A S177A did not alter myogenic tone at 60 mmHg or depolarization-induced vasoconstriction (60 mmol/L $K^+$) (**Figure 4-14D**). These data indicate that Rab11A S177 contributes to ET-1-induced vasoconstriction.

## Surface Trafficking of β1 Subunit Is Impaired in Myocytes of SP-SHRs Arteries

MAPs were 123±3 and 190±4 mmHg in WKY rats and SP-SHR, respectively ($p<0.05$). Western blotting revealed that BKα and β1 subunit total protein were similar in the cerebral arteries of WKYs and SP-SHR (**Figure 4-15A, B**). Arterial biotinylation experiments indicated BKα and β1 proteins were similarly distributed in cerebral arteries of WKYs and SP-SHR with >85 % of BKα present in the plasma membrane and >90 % of β1 located intracellularly (**Figure 4-15A, B**). These data indicate that BKα and β1 subunit total protein and cellular distribution are similar in unstimulated cerebral arteries of WKYs and SP-SHR.

We tested the hypothesis that trafficking of BK channel subunits induced by vasoactive stimuli is altered in cerebral arteries of SP-SHR during hypertension. Responses were measured to SNP, a nitric oxide donor, and membrane depolarization, which was produced by increasing extracellular $K^+$ to either 30 or 60 mM (10 min each). SNP, 30 mM $K^+$ and 60 mM $K^+$ increased surface β1 protein ~2.2-, 2.3- and 2.5- fold, respectively, in WKY arteries (**Figure 4-15A, B**). In contrast, SNP, 30 mM $K^+$ or 60 mM $K^+$ did not alter surface β1 protein levels in SP-SHR arteries (**Figure 4-15A, B**). SNP, 30 mM $K^+$ or 60 mM $K^+$ (10 min) did not alter surface levels of BKα in both WKY and SP-SHR arteries (**Figure 4-15A, B**). These data indicate that SNP- and depolarization-induced β1 subunit surface trafficking is functional in WKY rat arteries, but is blocked in myocytes of SP-SHR arteries.

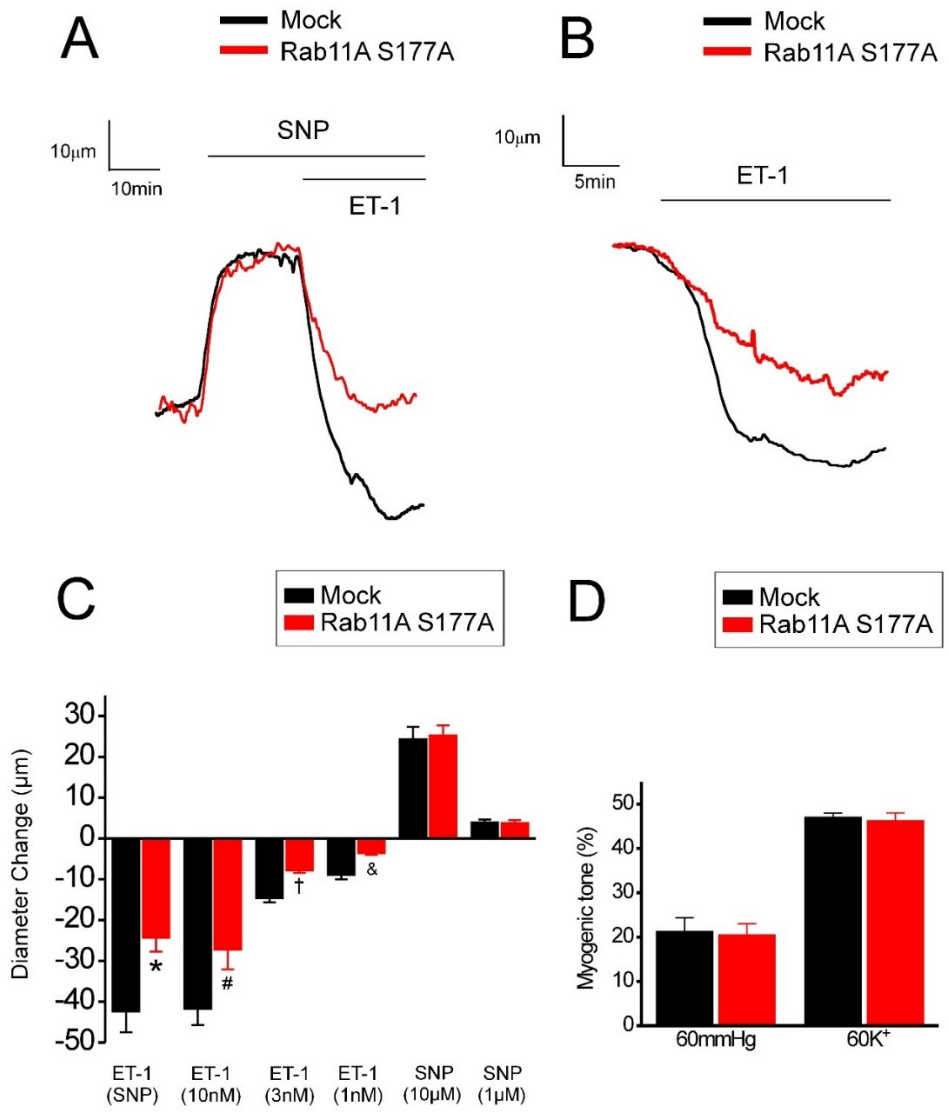


**Figure 4-14. ET-1 stimulates vasoconstriction by inhibiting surface trafficking of β1 subunits in pressurized arteries.**

*Notes:* (A) Representative diameter recordings of pressurized mock-transfected and Rab11A S177A vector-transfected arteries at 60 mmHg illustrating dilation to SNP (10 μmol/L) and constriction to ET-1 (10 nmol/L) applied in the presence of SNP. (B) Representative diameter traces from arteries at 60 mmHg, illustrating constriction to ET-1 (10 nmol/L). (C) Mean data illustrating diameter changes in response to: ET-1 (10 nmol/L) applied in the presence of SNP (10 μmol/L), ET-1 alone (1, 3 and 10 nmol/L) and SNP alone (1 and 10 μmol/L) at 60 mmHg. (n=6 for each group) *, #, † and & illustrate $p<0.05$ vs. the mock group in the same condition. (D) Mean data (n=6) of myogenic tone measured at 60 mmHg and in 60 mmol/L $K^+$.

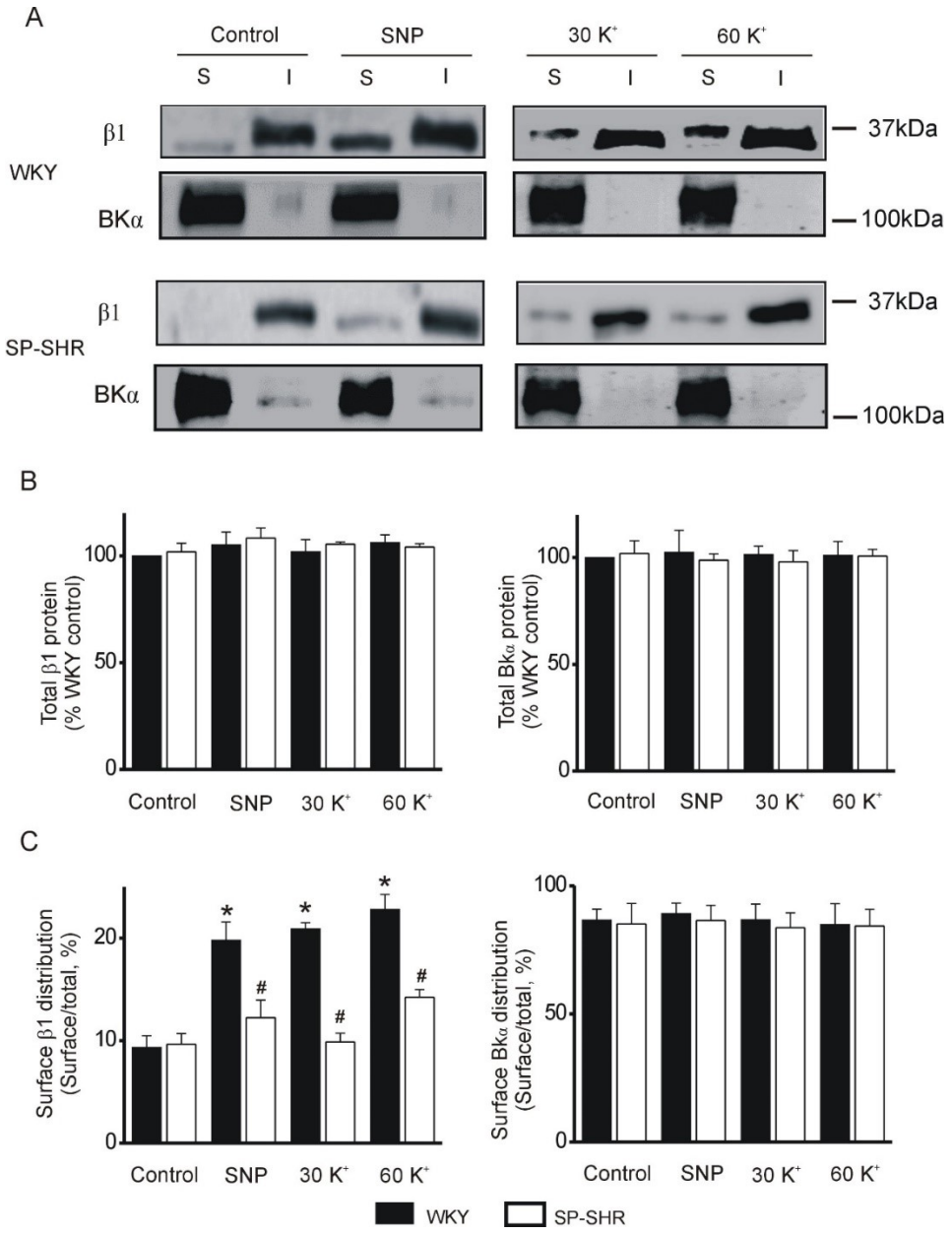


**Figure 4-15. NO-induced or depolarization-induced β1 subunits trafficking is impaired in spontaneous hypertensive rats.**

*Notes:* (A) Representative Western blots showing surface (S) and intracellular (I) β1 subunits and α subunits. Sodium nitroprusside (SNP, 10 μmol/L) (B) Total BKα and β1 protein (n=5) compared to WKY control. (C) Surface distribution of β1 subunits and α subunits (n=5). * $p<0.05$ vs. control in WKY; # $p<0.05$ vs. the same condition in WKY.

## NO-induced Activation of BK Channel Is Reduced in Myocytes of SP-SHR Due to Impaired β1 Subunit Trafficking

To investigate the functional consequence of β1 subunit trafficking inhibition, BK channel $P_O$ was measured in inside-out patches pulled from cerebral artery myocytes. $P_O$ was measured at -40mV, a physiological membrane potential, or at +80mV to stimulate maximal channel activity, using a free intracellular $Ca^{2+}$ concentration of 10 μM.

At -40 mV, the mean $P_O$ of BK channels in patches from control WKY and SP-SHR myocytes was similar (**Figure 4-16A, B**). Lithocholate, an activator of β1 subunit-containing BK channels, was applied directly to inside out-patches. Lithocholate increased BK channel $P_O$ ~2.4-fold in patches pulled from WKY rat myocytes but did not alter $P_O$ in patches from SP-SHR myocytes (**Figure 4-16A, B**). Myocytes were exposed to SNP (10 mins) after which the $P_O$ of BK channels was measured in inside-out patches in the absence of SNP. Myocyte SNP-treatment increased the $P_o$ of BK channels ~2.5-fold in patches from WKYs but did not alter the $P_O$ in those from SP-SHRs (**Figure 4-16A, B**). Lithocholate increased BK channel $P_O$ in SNP-treated WKY rat myocytes such that it was ~4.1-fold higher than in the control condition (**Figure 4-16A, B**).

In contrast, lithocholate did not activate BK channels in SNP-treated SP-SHR myocytes (**Figure 4-16A, B**). These data suggest that in WKY rat myocytes, some β1 subunits are associated with surface BK channels and that NO stimulates an increase in the amount of surface β1 subunits, which increases BK channel $P_O$ and activation by a β1 ligand. In contrast, in SP-SHR myocytes, surface β1 subunits do not appear to be associated with BKα and that NO does not stimulate β1 subunit surface trafficking to activate BK channels.

## BIM, a PKC Inhibitor, Enables SNP-induced β1 Subunit Surface Trafficking in SP-SHR Arteries

We hypothesized that dysfunctional β1 protein in SP-SHR was due to activation of PKC pathway. To evaluate the mechanism underlying inhibited β1 subunit trafficking, WKY and SP-SHR arteries were exposed to SNP with or without BIM, a PKC inhibitor. SNP alone increased surface β1 protein in WKY arteries but did not stimulate β1 protein surface trafficking in SP-SHR arteries (**Figure 4-17A, B**). BIM alone did not alter surface β1 protein in both WKY and SP-SHR arteries (**Figure 4-17A, B**). SNP similarly increased surface β1 when applied in either the absence or the presence of BIM (**Figure 4-17A, B**).

In contrast, BIM enabled SNP to increase surface β1 subunits ~2.7-fold in SP-SHR arteries (**Figure 4-17A, B**). Indeed, in the presence of BIM, SNP-induced surface trafficking of β1 subunits was the same as in WKY arteries (**Figure 4-17A, B**). BKα distribution was not altered by SNP or BIM applied either alone or in combination in WKY and SP-SHR arteries (**Figure 4-17A, B**). These data indicate that PKC inhibition

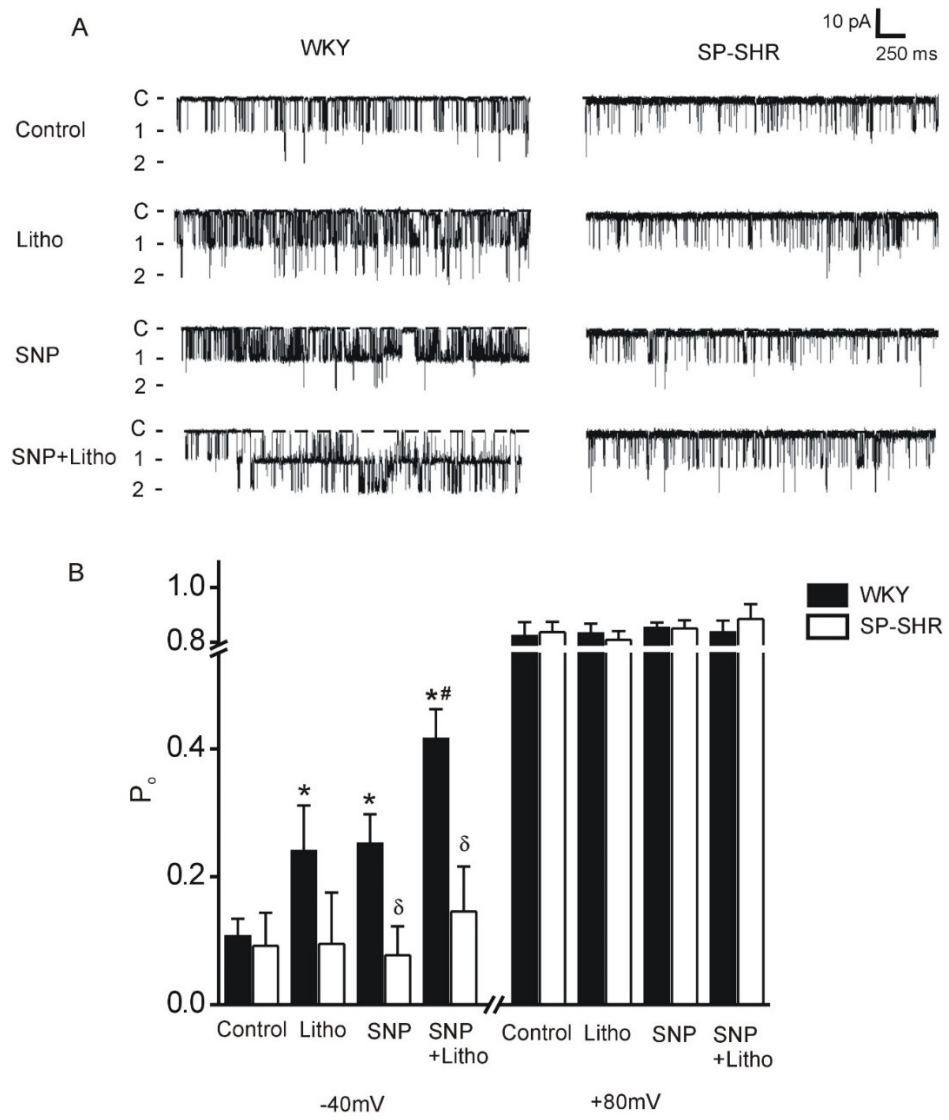


**Figure 4-16. NO-induced activation of BK channel is reduced in SP-SHR.**

*Notes:* (A) Representative traces of BK channels exposed to lithocholate (Litho, 150 μM) in inside-out patches from SNP (10 μM, 10min)-treated WKY and SP-SHR myocytes. SNP, (10 μM), BIM (10 μM, 1h). (B) Mean Po of BK channels under indicated conditions (n=6). * $p<0.05$ vs. control in WKY; # $p<0.05$ vs. SNP in WKY; δ $p<0.05$ vs. the same condition in WKY.

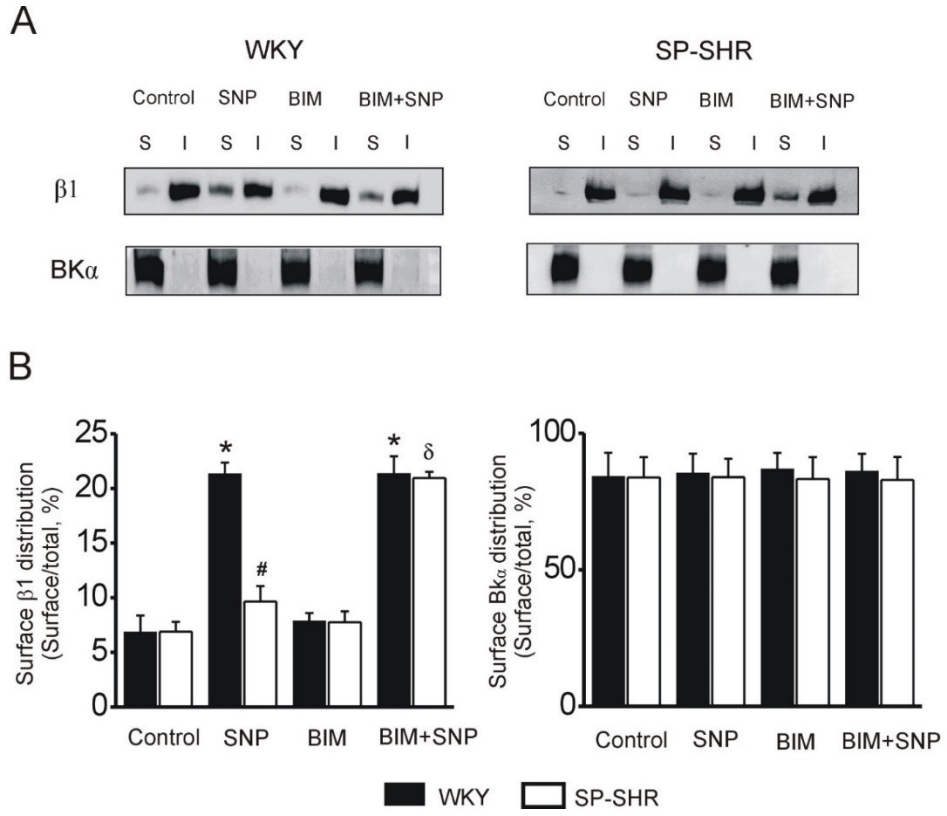


**Figure 4-17. NO-induced β1 subunits trafficking is attenuated via PKC activation in cerebral arteries of SP-SHR.**

*Notes:* (A) Representative Western blots showing surface (S) and intracellular (I) β1 subunits and α subunits under indicating conditions: SNP (10 μM, 10mins), BIM (10 μM, 1h), SNP(10 μM, 10mins)+BIM(10 μM, 1h). (B) Mean data of surface α and β1 proteins from biotinylation experiment (n=5). * $p<0.05$ vs. control in WKY; # $p<0.05$ vs. SNP in WKY; δ $p<0.05$ vs. control in SP-SHR.

does not alter the trafficking of β1 subunits by NO in WKY rat arteries, but restores NO-induced β1 subunit trafficking in SP-SHR arteries.

## Rab11A S177A Restores NO-induced β1 Subunit Surface Trafficking in SP-SHR Arteries

ET-1 activates PKC, which phosphorylates Rab11A at S177, leading to inhibition of β1 subunit surface trafficking (Zhai, Leo et al. 2017). Given that BIM restored β1 subunit trafficking in SP-SHR arteries, we evaluated the function of Rab11A S177A. Arteries were transfected with an empty vector (mock), or vectors that express either wild-type Rab11A (Rab11A WT) or Rab11A S177A, a phosphorylation-deficient Rab11A mutant. Vectors encoding Rab11A WT or Rab11A S177A similarly increased Rab11A total protein to ~162.6% or ~160.3% of the mock group (**Figure 4-18A, B**).

SNP similarly stimulated a ~3-fold increase in surface β1 subunits in WKY arteries overexpressing either Rab11A WT or Rab11A S177A (**Figure 4-18C, D**). SNP-induced surface trafficking of β1 subunits was impaired in SP-SHR cerebral arteries transfected with Rab11A WT (**Figure 4-18C, D**). In contrast, Rab11A S177A restored SNP-induced β1 surface trafficking in SP-SHR arteries (**Figure 4-18C, D**). These data suggest that PKC-mediated phosphorylation of Rab11A S177 inhibits β1 surface trafficking in cerebral arteries of SP-SHR.

## Rab11A S177A Restores BK Channel Activation in SP-SHR Arterial Myocytes

To examine the modulation of BK channel $P_O$ by Rab11A S177, myocytes were isolated from WKY or SP-SHR arteries overexpressing Rab11A or Rab11A S177A. Myocytes were exposed to SNP (10 mins), after which inside-out patches were pulled and BK channels recorded in the absence of SNP. BK channel $P_O$ was similar in WKY rat myocytes expressing either Rab11A or Rab11A S177A (**Figure 4-19A, B**).

In patches from SP-SHR arteries expressing Rab11A WT, mean BK channel $P_O$ was ~60% of that in WKY myocytes expressing the same construct (**Figure 4-19A, B**). The expression of Rab11A S177A increased BK channel $P_O$ in SP-SHR myocytes, such that it was the same as in WKY myocytes under the same condition (**Figure 4-19A, B**).

The application of lithocholate to inside-out patches similarly increased BK channel $P_O$ ~1.8-fold in WKY rat myocytes expressing either Rab11A WT or Rab11A S177A (**Figure 4-19A, B**). Lithocholate did not increase BK channel $P_O$ in SP-SHR myocytes expressing Rab11A WT (**Figure 4-19A, B**).

In contrast, Rab11A S177A expression restored lithocholate-induced activation of BK channels in SNP-treated SP-SHR myocytes, such that $P_O$ was the same as in WKY rat myocytes under the same condition (**Figure 4-19A, B**). These data indicate that

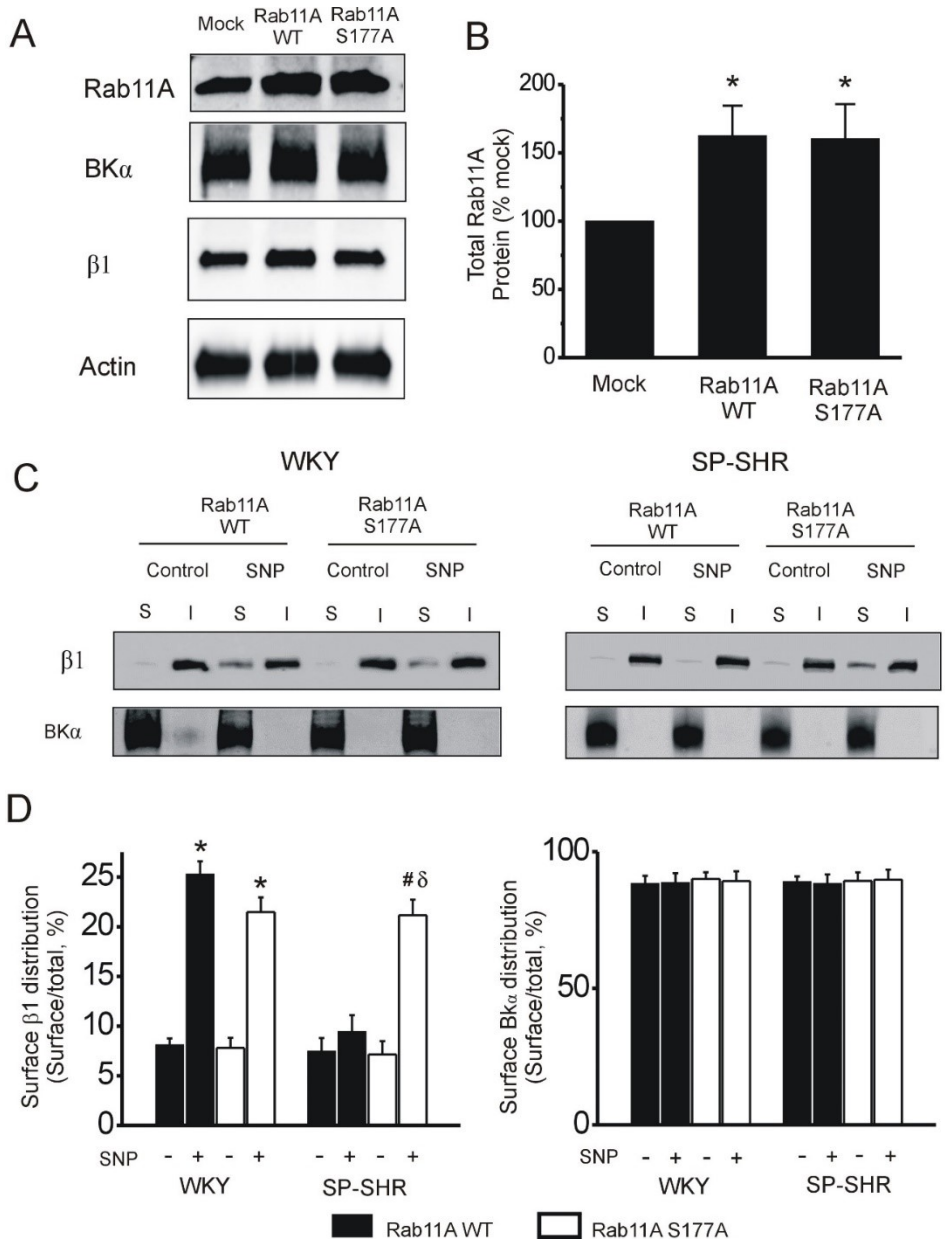


**Figure 4-18. Rab11A S177A restores SNP-induced surface-trafficking of β1 subunits in cerebral arteries of SP-SHRs.**

*Notes:* (A) Representative western blots of Rab11A, BKα, β1 subunits and actin. (B) Mean data of Rab11A protein compared with control (n=5). * $p<0.05$ vs. mock. (C) Representative Western blots showing surface (S) and intracellular (I) β1 subunits and α subunits after transfection under each indicating condition. (D) Mean data of surface protein of β1 subunits and α subunits. (n=5) * $p<0.05$ vs. control (no SNP) in WKY; # $p<0.05$ vs. control (no SNP) in SP-SHR; δ $p<0.05$ vs. SNP in SP-SHR Rab11A WT.

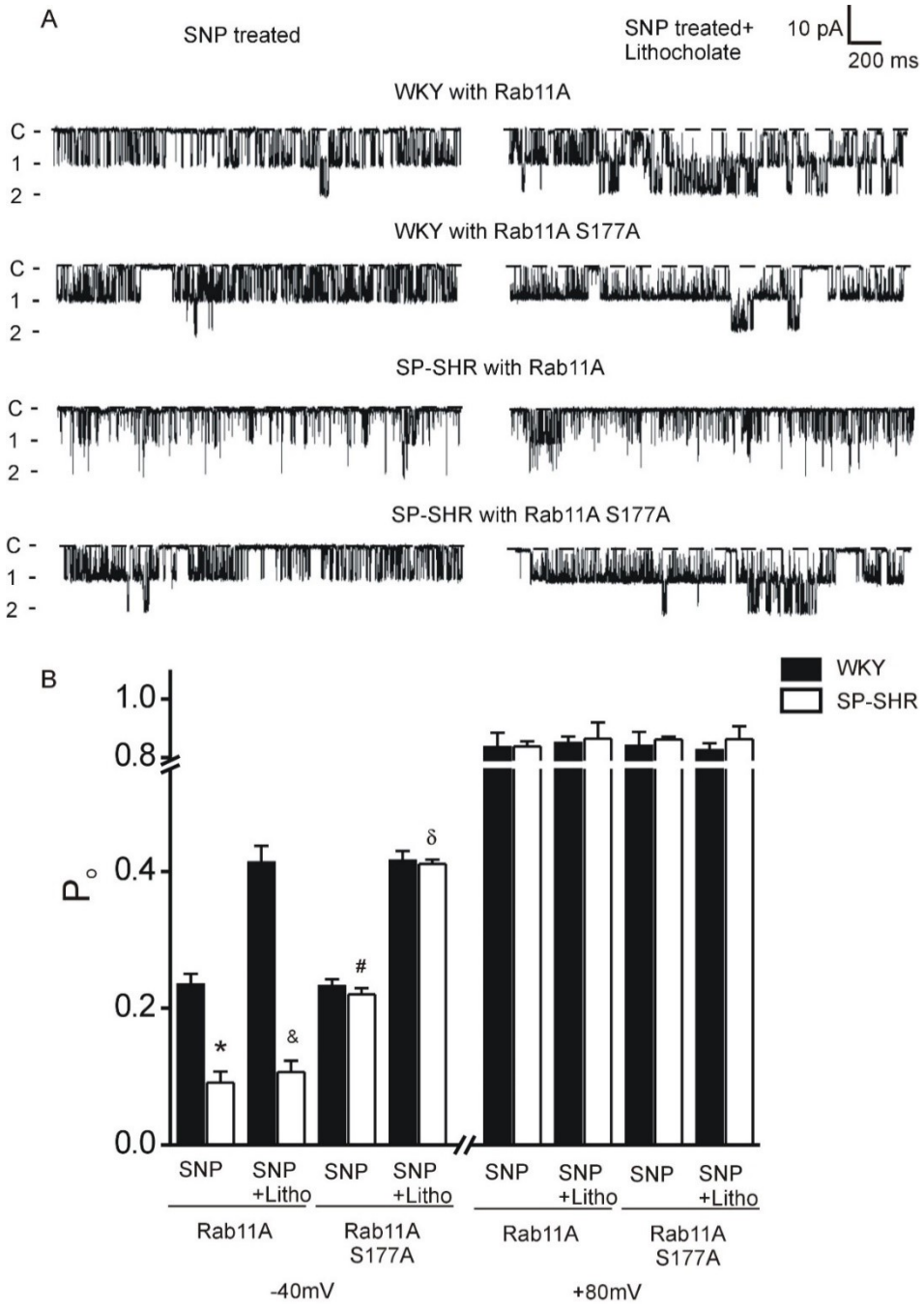


**Figure 4-19. Rab11A S177A increases mean Po via stimulating β1 subunits trafficking in SP-SHR.**

*Notes:* (A) Representative traces recording BK channels from smooth muscle cells transfected with Rab11A WT or Rab11A S177A under indicating conditions. (B) Mean Po of inside-out patches after transfection (n=6). * $p<0.05$ vs. SNP in WKY Rab11A WT; & $p<0.05$ vs. SNP+litho in WKY Rab11A WT; # $p<0.05$ vs. SNP in SP-SHR Rab11A WT; δ $p<0.05$ vs. SNP+litho in SP-SHR Rab11A WT.

Rab11A S177A expression restores SNP-induced BK channel activation, supporting other evidence here that PKC-mediated phosphorylation of Rab11A S177 blocks BK channels in SP-SHR myocytes.

### Impaired β1 Subunit Trafficking Attenuates BK Channel Functionality in Pressurized SP-SHR Arteries

The functional impact of dysfunctional β1 trafficking was investigated by measuring the contractility of pressurized cerebral arteries. At an intravascular pressure of 60 mmHg, endothelium-denuded SP-SHR arteries developed 36.1±1.8 % tone compared with 23.9 ±4.0 % tone in WKY arteries, or ~1.5-fold more (n=, 6; $P<0.05$).

SNP and lithocholate increased the mean diameter of SP-SHR arteries by ~4 and 6 μm, respectively, responses that were ~26 and 48 %% of those in WKY rat arteries (**Figure 4-20A, B**). SNP increased lithocholate-induced dilation from~ 6 to 20 μm in WKY rat arteries and from ~3 to 7 μm in SP-SHR arteries. Thus, lithocholate-induced vasodilation in the presence of SNP was ~2.8-fold more in WKY than SP-SHR arteries (**Figure 4-20B**). BIM increased SNP-induced vasodilation in SP-SHR arteries to the same as in WKY rat arteries (**Figure 4-20B**).

BIM also increased lithocholate-induced vasodilation obtained in the presence of SNP in SP-SHR arteries to the same as that in WKY rat arteries (**Figure 4-20B**). In contrast, BIM alone did not alter WKY rat or SP-SHR artery diameter (**Figure 4-20B**). BIM also did not alter vasodilation to SNP or lithocholate in WKY rat arteries (**Figure 4-20B**) These data support our biochemical evidence that dysfunctional mobilization of myocyte BK channel β1 subunits greatly attenuates BK channel functionality through PKC activation in SP-SHR arteries.

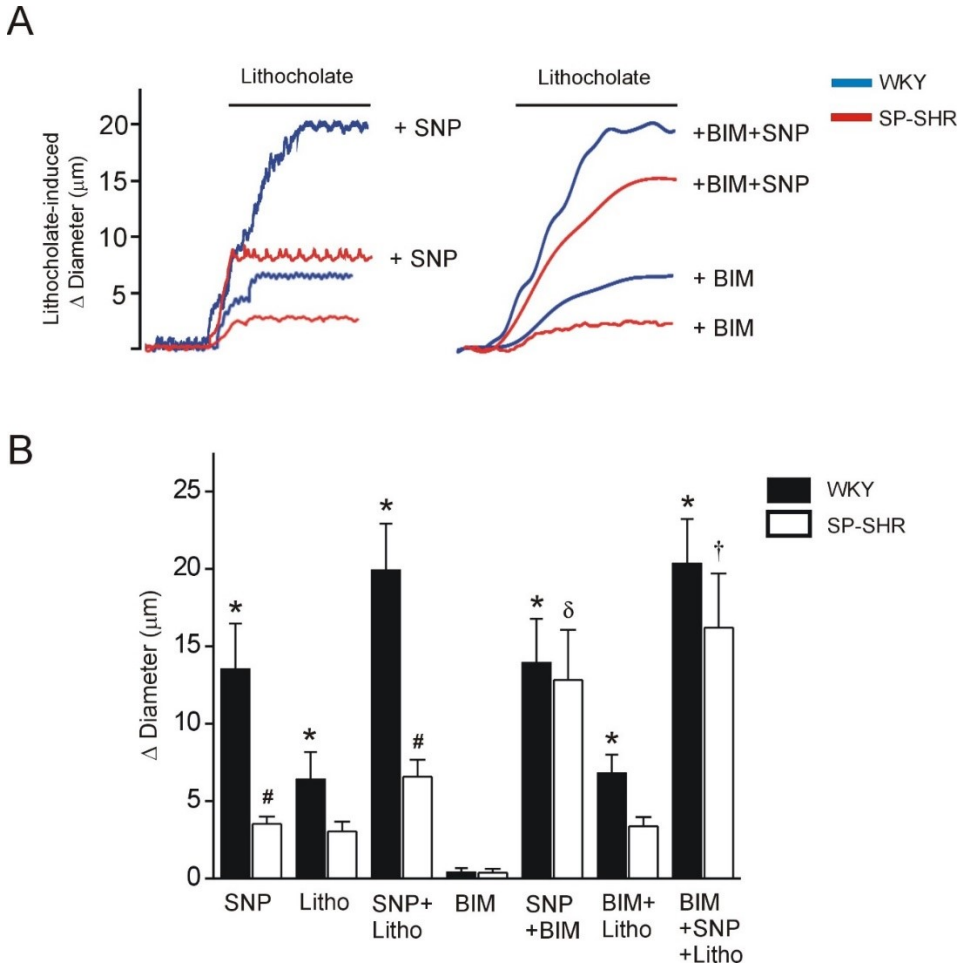


**Figure 4-20. Activation of PKC dampens NO-mediated vasodilation in SP-SHR.**

*Notes*: (A) Representative traces of changes in arterial diameter from WKY rats (blue) and SP-SHR (red). (B) Mean data illustrating diameter changes (n=6 for each). * $p<0.05$ vs. control in WKY; # $p<0.05$ vs. WKY in the same condition; δ $p<0.05$ vs. SNP in SP-SHR; † $p<0.05$ vs. SNP + Litho in SP-SHR.

# CHAPTER 5. DISCUSSION*

## Summary of Findings

Here, the aim of this study was to investigate the mechanism how β1 subunits trafficking regulated by vasoconstrictors. Our data indicate that endothelin-1 inhibited β1 subunits anterograde trafficking to the cell surface via activation of PKC. The inhibitory effect of β1 subunits anterograde trafficking by ET-1 reduces the open probability of BK channel. It has been shown that β1 subunits are co-localized with Rab11A positive recycling endosome. SNP increases active Rab11 whereas ET-1 reduces active Rab11. Active Rab11A facilitates β1 subunits trafficking towards the cell surface. Activation of PKC phosphorylates Rab11A, particularly S177, to inhibit the activity of Rab11A. Loss activity of Rab11A fails to stimulate β1 subunits trafficking to the cell surface. Rab11A S177A reduces the open probability and the transient outward currents of BK channels. Moreover, Rab11A S177A attenuates ET-1 induced vasoconstriction. Thus, we conclude that ET-1 inhibits β1 subunits anterograde trafficking by phosphorylating Rab11A on S177 via activation of PKC.

We also tested the hypothesis that hypertension is associated with altered trafficking of BK channel subunits in myocytes of cerebral arteries using SP-SHRs as a disease model and WKY rats as a control. Our data show that stimulated surface trafficking of β1 subunits by NO or membrane depolarization is inhibited in arterial myocytes of SP-SHRs. PKC inhibition or expression of Rab11AS177A, a PKC phosphorylation-deficient mutant, restores β1 subunit surface-trafficking in SP-SHR arteries. BK channel activation by NO is attenuated due to the impaired trafficking of β1 subunits in arterial myocytes of SP-SHRs. BK channel activation by NO is restored by Rab11AS177A in SP-SHR myocytes. PKC inhibition also enables NO-induced vasodilation by reestablishing the ability of NO to increase the abundance of β1 subunits associated with surface BKα. These data demonstrate that spontaneously active PKC blocks the ability of NO to stimulate β1 subunit surface trafficking in myocytes of SP-SHR, thereby preventing BK channel activation and vasodilation in cerebral arteries of SP-SHR.

## ET-1 Inhibits NO or Depolarization-induced Surface Trafficking of β1 Subunits in Myocytes of SD Rats

Previous studies have shown that SNP and membrane depolarization, stimulate anterograde trafficking of recycling endosomes via PKG or ROCK pathway, respectively.

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* Portions of this chapter adapted with permission. Zhai, X., M. D. Leo and J. H. Jaggar (2017). "Endothelin-1 Stimulates Vasoconstriction Through Rab11A Serine 177 Phosphorylation." Circ Res **121**(6): 650-661.

β1 subunits was delivered to the cell surface in arterial myocytes. (Leo, Bannister et al. 2014, Leo, Zhai et al. 2017) β1 subunits has been shown to increase apparent $Ca^{2+}$sensitivity of BK channels when β1 subunits are associated with pore forming α subunits. β1 subunits are dynamically trafficked back and force from the cell surface to intracellular endosome. SNP, the vasodilator, is stimulated the anterograde trafficking of β1 subunits to cell surface. This led us to propose that the vasoconstrictors act the opposite way against vasodilators. Because only ~8% of β1 subunits are presented at cell surface at physiological level, ET-1 did not significantly reduce the surface expression. However, the functional studies from myography did show the ~20% attenuation of ET-1 vasoconstriction. Although ET-1 does not significantly reduce surface expression of β1 subunits, ET-1 retains β1 subunits in the intracellular compartment resulting in vasoconstriction. But ET-1 significantly reduced NO-induce β1 subunits anterograde trafficking. BIM-1, the PKC inhibitor, ablated the inhibitory effect of ET-1 on β1 subunits anterograde trafficking.

Studies has shown that PKG and PKC regulates the open probability of BK channels. Activation of PKG by vasodilators increases the open probability of BK channels by stimulating β1 subunits trafficking to the cell surface. Activation of PKC by long period of time decrease BK channel currents due to internalization and degradation of BKα channels. Our data indicate that that activation of PKC also reduces the open probability of BK channel by blocking β1 subunits trafficking to the cell surface.

## Rab11A Protein Functions in β1 Subunits Trafficking

Rab protein belongs to small GTPases that control protein trafficking in various types of cells. (Zerial and McBride 2001, Stenmark 2009) β1 subunits have been shown to be co-localized with Rab11A in recycling endosome in smooth muscle cells.(Leo, Bannister et al. 2014) β1 subunits was not stimulated by SNP in Rab11A knockdown smooth muscle cells. (Leo, Bannister et al. 2014) It still remains unknown that how activation of PKG stimulates protein trafficking of β1 subunits. It is possible that PKG directly phosphorylate Rab11A which then activate Rab11A. Another possibility could be PKG activate other signaling protein which then activates Rab11A indirectly. We could not exclude that PKG does not alter Rab11A activity. Here, our data show that Rab11 activity is increased by NO in arterial myocytes. It indicates that PKG activates Rab11A. We also show that ET-1 reduces Rab11A activity, demonstrating that differential regulation of Rab11A activity controls surface β1 trafficking. On the other hand, PKC may control upstream proteins or directly phosphorylate Rab11A so that it inhibits Rab11A activity. To test these possibilities, bioinformatics showed that five possible sites in Rab11A might be phosphorylated by PKC, whereas S177 has the highest possibility. To test S177 function, we transfected cerebral arteries with Rab11A S177A. Rab11A S177A reduced ET-stimulated phosphorylation of Rab11A in cerebral arteries. The data indicate that Rab11A S177 is phosphorylated by protein kinase C which results in a decrease in activity of Rab11A. ET-1-reduced surface trafficking of β1 protein was also attenuated by Rab11A S177A. On the other hand, NO-induced surface trafficking of β1protein was not changed by Rab11A S177A. The explanation might be that Rab11A is

activated by NO via a different pathway that is not involved in S177. Previous finding suggested that a PKC activator (phorbol myristate acetate) activated Rab11 phosphorylation in HeLa cells expressing Rab11.(Pavarotti, Capmany et al. 2012) Moreover, serotonin (5-HT) activated PKC which induced sequestration of 5-HT receptors into a Rab11-positive endosome in HEK293 cells.(Idkowiak-Baldys, Baldys et al. 2009) In summary, our data indicate that ET-1 stimulates PKC-mediated phosphorylation of Rab11A serine 177, leading to a decrease in Rab11A activity and a reduction in surface β1 subunits in arterial myocytes.

### PKC Pathway Is Involved in β1 Subunits Trafficking

Vasoregulatory stimuli regulate activity of BK channel through protein kinases in arterial myocytes.(Barman 1999, Schubert, Noack et al. 1999, Betts and Kozlowski 2000, Taguchi, Kaneko et al. 2000, Barman, Zhu et al. 2004, McNair, Salamanca et al. 2004, Rainbow, Norman et al. 2009) Previous studies showed that the whole-cell BK currents was inhibited by ET-1 in rat renal artery myocytes, but the mechanisms involved were not fully understood.(Betts and Kozlowski 2000) The inside-out membrane patche data showed that the activity of BK channels was increased by PKA and PKG in arterial myocytes.(Robertson, Schubert et al. 1993, White, Kryman et al. 2000, Schubert and Nelson 2001) In contrast, BK channels was inhibits by PKC in inside-out patches.(Bonev, Jaggar et al. 1997, Zhou, Wulfsen et al. 2010) The frequency of $Ca^{2+}$ sparks was also modulated by PKA, PKG, and PKC to control BK channel activity indirectly. (Jaggar, Porter et al. 2000) Here, we showed that a vasoconstrictor (ET-1) decreased anterograde trafficking of β1 subunits and surface expression of β1 proteins to inhibit BK channels. Previous data similarly suggested that an increase of mean Po in BK channel was caused by an increase in surface β1 protein by NO stimulation. (Leo, Bannister et al. 2014) In contrast, mean Po of BK channel was inhibited by ET-1-induced PKC activation in membrane patches. We showed that ET-1-inhibition of BK channel activity was reversed by BIM and Rab11A S177A. It supported the biochemistry data that PKC-induced phosphorylation of Rab11A S177 inhibited β1 anterograde trafficking in smooth muscle cells.

### Rab11A S177A Enables Transient Outward Currents in Presence of ET-1

$Ca^{2+}$ sparks are generated by sarcoplasmic reticulum which activate several nearby BK channels in arterial myocytes.(Jaggar, Porter et al. 2000) To study how $Ca^{2+}$ sparks was modulated by Rab11A S177, we measured transient BK currents by whole cell configuration in smooth muscle cells. Previous study showed similar data that SNP increased both mean AUC and frequency of transient BK current. (Porter, Bonev et al. 1998) Here, we showed that Rab11A S177A did not alter the SNP-induced increase in transient BK current frequency or AUC. It indicated that PKG activates transient BK current via S177-independent mechanism. ET-1 inhibited an increase in transient BK current frequency and mean AUC induced by NO in control myocytes. However, the ET-1-induced reduction in mean AUC was reversed by Rab11A S177A in transfected

arteries. It suggested that the effective coupling of BK channels to $Ca^{2+}$ sparks was attenuated by ET-1 mediated activation of PKC. Furthermore, S177A did not alter the ET-1 mediated decrease in transient BK current frequency. $Ca^{2+}$ spark frequency determines transient BK current frequency because $Ca^{2+}$ sparks activate a transient BK current in rat cerebral artery myocytes. (Jaggar, Porter et al. 2000) It indicated that Rab11A S177 did not modify $Ca^{2+}$ spark frequency so that it did not change frequency of transient outward currents. Our data indicate that ET-1 phosphorylates Rab11A at S177 in order to inhibit BK channel transient BK currents.

### Rab11A S177A Attenuates ET-1-induced Vasoconstriction

ET-1 activates protein kinase C which phosphorylates myosin light chain to induce vasoconstriction. (Kamm and Stull 1985, Adam, Milio et al. 1990, Somlyo and Somlyo 2003, Davenport, Hyndman et al. 2016) Here, our data indicated that ET-1-induced vasoconstriction was impaired by Rab11A S177 in the presence of SNP. Moreover, vasoconstriction to various concentration of ET-1 was also reduced by Rab11A S177A overexpression in cerebral arteries. These data also were consistent with the data that membrane depolarization induced by intravascular pressure was attenuated by ET-1 by inhibiting β1 surface trafficking. (Leo, Zhai et al. 2017) In contrast, membrane depolarization (60 mmol/L $K^+$)-induced vasoconstriction was not altered by Rab11A S177A because 60 $K^+$ induced very potent vasoconstriction which might mask the effect of Rab11A S177A. Myogenic tone was not changed by Rab11A S177A either. It suggested that Rab11A S177A is not a non-specific inhibitor of vasoconstriction. In summary, our data demonstrate that ET-1 induces vasoconstriction by blocking β1 subunit surface trafficking and in turn inhibiting BK channel activity.

### Surface Trafficking of β1 Total Proteins in SP-SHR Arteries

Cerebral arteries from hypertensive subjects are depolarized and constricted.(Harder, Brann et al. 1983, Harder, Smeda et al. 1985) Endothelium-dependent vasodilation, including that produced by NO, is attenuated in vessels from multiple hypertensive animal models.(Luscher, Aarhus et al. 1990, Cordellini 1999, Heitzer, Wenzel et al. 1999, Zhou, Kosaka et al. 2001, Yang, Gluais et al. 2004, Quaschning, Hocher et al. 2006, Jimenez, Lopez-Sepulveda et al. 2007, Choi, Allahdadi et al. 2011)

Given that BK channels regulate physiological arterial potential and contractility, we investigated whether pathological changes in BK channel trafficking alter arterial function during hypertension. We used SP-SHR, a rat genetic model of hypertension that is prone to stroke, to study the cellular distribution and regulation of BKα and β1 subunit total and surface abundance. Our data show that BKα and β1 total and surface proteins are similar in cerebral arteries of age-matched hypertensive SP-SHRs and normotensive WKY rat controls. Our data are in agreement with the findings of some, but not all, previous studies that measured either subunit message or total protein in cerebral arteries.

Consistent with our results, Pabbidi reported that BKα and β1 total proteins were similar in cerebral arteries of a hypertensive model of Fawn Hooded rats, when compared with their normotensive genetic controls (Pabbidi, Mazur et al. 2014). Other studies have reported that BKα mRNA is unaltered, but β1 mRNA was lower in cerebral arteries of angiotensin II-infused hypertensive Sprague-Dawley rats and mice and SHRs, when compared with their respective controls (Amberg, Bonev et al. 2003, Amberg and Santana 2003). Another study found that although BKα mRNA was similar, BKα protein was higher in cerebral arteries of SHR and WKYs (Liu, Hudetz et al. 1998). Some of these different findings may relate to the approaches used to measure BK channel subunit expression or the animal models of hypertension that were examined. Regardless, our data show that the altered functionality of BK channels described here in SP-SHR cerebral arteries is not due to a change in BKα or β1 total proteins.

**Rab11A-mediated B1 Subunits Trafficking in Myocytes of SP-SHRs**

We previously described that in unstimulated arteries, low levels of β1 subunits are trafficked to the surface via a Rab11A-independent mechanism (Leo, Bannister et al. 2014). Our data here suggest that the surface-trafficking of these basal β1 subunits is not altered in SP-SHR. NO and membrane depolarization stimulate Rab11A-dependent trafficking of β1 subunits to the plasma membrane in arterial myocytes, but through distinct signaling mechanisms: NO operates through PKG activation, whereas depolarization acts via the stimulation of ROCK (Leo, Bannister et al. 2014, Leo, Zhai et al. 2017). Regardless of the signaling mechanisms involved, surface-trafficked β1 proteins associate with plasma membrane-resident BKα, leading to channel activation (Leo, Bannister et al. 2014, Leo, Zhai et al. 2017).

Here, NO and membrane depolarization failed to surface-traffic β1 subunits in cerebral arteries of SP-SHRs, indicating the presence of an endogenous inhibitory mechanism. We recently described that endothelin-1 activates protein kinase C, which phosphorylates Rab11A at Serine 177. (Zhai, Leo et al. 2017) This mechanism reduces Rab11A activity and blocks β1 subunit surface trafficking in arterial myocytes (Zhai, Leo et al. 2017). Given that NO stimulates Rab11A, it leads us to hypothesize that activated PKC activation may prevent β1 trafficking in myocytes of SP-SHR (Zhai, Leo et al. 2017).

Data we provide here support this mechanism, including that BIM and Rab11AS177A, a mutant Rab11A that is unable to be phosphorylated by PKC at serine 177, enabled NO-induced trafficking of β1 subunits in SP-SHR arteries. In contrast, the overexpression of Rab11A did not reestablish β1 trafficking, indicating that the inhibitory mechanism was not specific to endogenous Rab11A and could not be overcome by increasing the amount of Rab11A. Identifying the mechanism by which PKC is spontaneously activated in arteries of SP-SHRs was not a focus of our study, but several possibilities exist. An increase in the expression, translocation and activity of PKC has been implicated in the pathogenesis of hypertension and one or more of these signaling

alterations may underlie PKC-mediated Rab11A inhibition in SP-SHR arterial myocytes (Salamanca and Khalil 2005).

Our data show that PKC is active in unstimulated arteries *in vitro*. The PKC-mediated inhibitory mechanism was also present in arteries that had been placed in serum-free media for 2 days. These data suggest that the mechanism of PKC activation occurs in the isolated arterial wall and that *in vivo* factors, such as circulating vasoconstrictors, are not required for this PKC activity and inhibition of β1 trafficking in arterial myocytes of SP-SHRs.

PKC can be activated by many different intracellular signal elements, including diacyglycerol, $Ca^{2+}$ and reactive oxygen species. Studies have shown that intracellular $Ca^{2+}$ concentration and $Ca^{2+}$ sparklet activity are increased in arterial myocytes during hypertension (Nieves-Cintron, Amberg et al. 2007, Joseph, Thakali et al. 2013). Hypertension is also associated with an increase in the bioavailability of vascular reactive oxygen species (Montezano, Dulak-Lis et al. 2015). One or more of these multiple mechanisms may activate PKC, leading to the inhibition of β1 trafficking in arterial myocytes of SP-SHRs.

## Regulation of BK Channel Activity by β1 Subunits Trafficking in Myocytes of SP-SHRs

BK channels formed from four α subunits can contain between one and four β1 subunits (Knaus, Garcia-Calvo et al. 1994, Wang, Ding et al. 2002). The α:β1-tetramer ratio shifts channel voltage- and $Ca^{2+}$ dependence, with an increase in the number of β1 subunits elevating channel activity(Wang, Ding et al. 2002). Here, we applied lithocholate directly to inside-out patches to evaluate the presence of channels containing β1 subunits. Although surface β1 protein was similar in unstimulated WKY and SP-SHR myocytes, lithocholate activated BK channels only in patches from WKY rats and not in patches from SP-SHRs. Thus, plasma membrane β1 subunits in unstimulated SP-SHR arterial myocytes do not activate BK channels. Explanations for this finding include that a pathological signaling mechanism prevents low levels of β1 subunits from either activating or physically associating with BKα in in SP-SHR myocytes.

SNP increased both BK channel $P_O$ and lithocholate-induced channel activation in excised patches from WKY rat myocytes, indicating that NO increased the abundance of β1 subunits associated with BKα. In contrast, SNP did not activate BK channels or enable lithocholate-induced BK channel activation in SP-SHR myocytes, consistent with other data here that NO did not traffic β1 subunits to the surface in these cells. The overexpression of Rab11A did not enable NO- or lithocholate-activation of BK channels in SP-SHR arterial myocytes. In contrast, Rab11A S177A reestablished both NO- and lithocholate-activation of BK channels. These data suggest that PKC acting through Rab11A S177 inhibits β1 subunit surface-trafficking, thereby preventing NO from activating BK channels.

## Spontaneous Activated PKC Inhibits Vasodilation of SP-SHR Arteries

Functional data also support the signaling mechanism identified here using biochemistry and electrophysiology. In pressurized WKY rat arteries, SNP and lithocholate both stimulated dilation, SNP increased lithocholate-induced vasodilation and BIM did not alter these responses. In contrast, in SP-SHR arteries, SNP and lithocholate-induced dilations were small, SNP did not increase lithocholate-induced dilation, BIM increased SNP-induced vasodilation and enabled SNP to augment dilation to lithocholate. Importantly, BIM increased SNP-induced vasodilation such that it was the same as in WKY rat arteries. These data indicate that PKC-mediated inhibition of β1 subunit trafficking underlies dysfunctional NO-induced vasodilation in SP-SHR cerebral arteries.

As discussed above, studies have reported either no change or alterations in the abundance of BK channel subunits in hypertensive animal models. The upregulation of BK currents in arterial myocytes during SHRs is reported to elicit compensatory vasodilation (Liu, Hudetz et al. 1998). In contrast, a reduction in β1 subunits has been described to decrease BK channel activity, producing vasoconstriction in angiotensin II-infused hypertensive Sprague-Dawley rats and mice and SHRs (Amberg, Bonev et al. 2003, Amberg and Santana 2003, Nieves-Cintron, Amberg et al. 2007). Our data indicate that BKα and β1 total protein is unaltered and myogenic tone is higher in pressurized arteries of SP-SHRs than WKY rats. It was not a focus of this study to investigate the underlying mechanisms for increased myogenic tone and whether BK channels contribute to this vasoconstriction or oppose it. BIM alone did not alter the diameter of either WKY rat or SP-SHR arteries, suggesting that the net effect of PKC activation does not contribute to the increase in myogenic tone. Other mechanisms, including those mediated through voltage-dependent $K^+$ ($K_V$) and $Ca_V1.2$ channels contribute to this pathological vasoconstriction (Joseph, Thakali et al. 2013). Our data show that NO-induced vasodilation is attenuated due to PKC-mediated inhibition of β1 subunit trafficking in cerebral arteries of SP-SHRs.

## Conclusion

In summary, we show that ET-1 activates PKC, which phosphorylates Rab11A S177, leading to a decrease in Rab11A activity and a reduction in β1 subunit surface trafficking. The ET-1-induced reduction in β1 subunit surface delivery inhibits BK channels and transient BK currents, leading to vasoconstriction. In contrast, NO activates Rab11A and increases surface β1 subunits through a Rab11A S177-independent mechanism. In summary, we show that differential regulation of Rab11A by ET-1 and NO controls BK channels and contractility in arterial myocytes. (**Figure 5-1**)

We also show that that activated PKC blocks the stimulated surface trafficking of β1 subunits, which prevents BK channel activation in arterial myocytes of SP-SHRs.

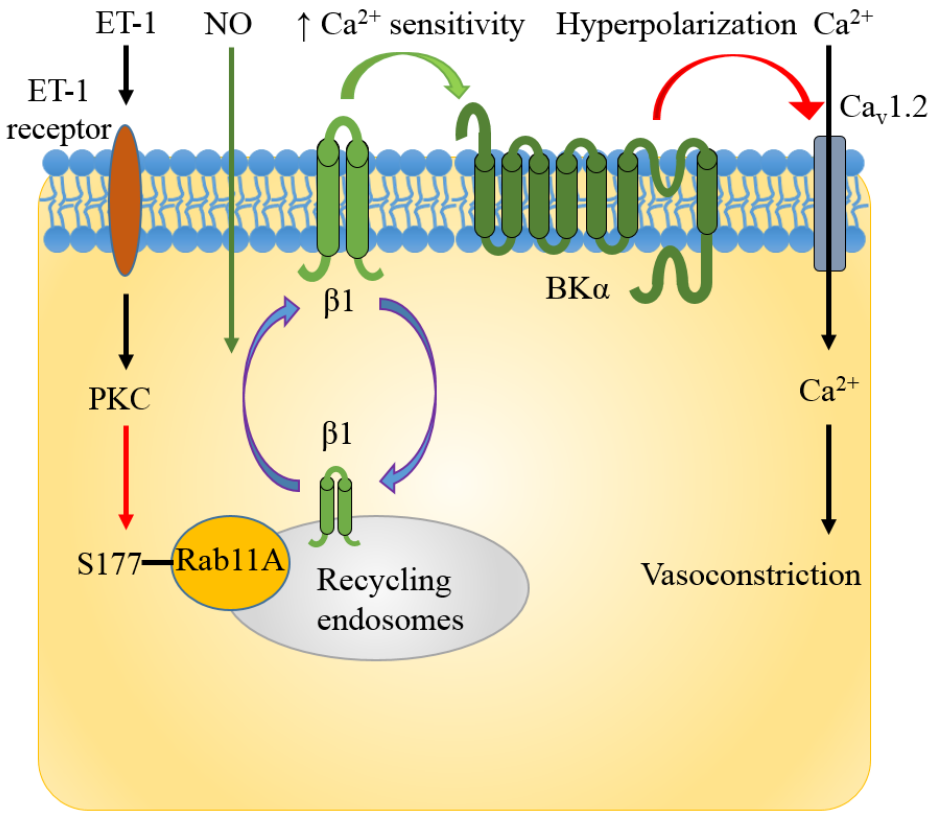


**Figure 5-1. Signaling pathways of the β1 subunit trafficking in arterial myocytes.**

*Notes:* Nitric oxide (NO) stimulates surface trafficking of β1 subunits via PKG activation. Endothelin-1 (ET-1) activates PKC to phosphorylate Ser177 on Rab11A, which inhibits NO-induced surface trafficking of β1 subunits towards cell surface. It reduces BK channel activity which in turn decreases inhibition of $Ca_v1.2$ channel and results in vasoconstriction.

Inhibition of this PKC-mediated inhibitory mechanism restores both stimulated surface trafficking of β1 subunits, BK channel activation and vasodilation. (**Figure 5-2**)

## Novelty and Significance

Large-conductance calcium ($Ca^{2+}$)-activated potassium channels (BK) are expressed in arterial smooth muscle cells and regulate contractility. β1 subunit anterograde trafficking is controlled by Rab11A, a Rab GTPase, and surface abundance can be increased by several stimuli that activate BK channels, including nitric oxide and membrane depolarization, leading to vasodilation. Whether vasoconstrictors modulate the surface trafficking of β1 subunits to inhibit BK channels is unclear.

We show that endothelin-1, a potent vasoconstrictor, stimulates protein kinase C, which phosphorylates Rab11A at serine 177, reducing Rab11A activity and Rab11A-dependent surface trafficking of β1 subunits. The endothelin-1-induced decrease in surface β1 subunits inhibits BK channels, resulting in vasoconstriction.

Arterial contractility is modulated by both vasoconstrictor and vasodilator stimuli, leading to changes in regional organ blood flow and systemic blood pressure. Several different ion channels are expressed in arterial smooth muscle cells, but whether regulated trafficking of these proteins controls contractility is poorly understood. In arterial myocytes, BK channel consists of BKα subunits and β1 subunits. Recent evidence indicates that BKα is primarily located in the plasma membrane, whereas β1 subunits are dynamic proteins whose localization can be modulated. It is unclear whether vasoconstrictors inhibit BK channels and stimulate contraction by reducing the surface abundance of β1 subunits and their association with surface BKα. We show that ET-1 stimulates protein kinase C-mediated phosphorylation of Rab11A, a Rab GTPase, at serine 177, which inhibits Rab11A and Rab11A-mediated surface trafficking of β1 subunits. This mechanism inhibits BK channels, leading to vasoconstriction. Our study describes a unique mechanism by which a vasoconstrictor inhibits BK channels to stimulate contraction and identify Rab11A serine 177 as a potential target site by which to modulate arterial contractility.

Findings of our study may be relevant for human cardiovascular diseases. Single nucleotide polymorphisms (SNPs) in BKα and β1 genes contribute to human cardiovascular diseases, including hypertension (Kohler 2010). More than 140 SNPs have been reported in or nearby the β1 subunit gene. An E65K polymorphism in β1 produces a "gain-of-function" mutant which increases BK channel $Ca^{2+}$-sensitivity and is associated with lower prevalence for diastolic hypertension (Fernandez-Fernandez, Tomas et al. 2004). BK channel $Ca^{2+}$-sensitivity is lower in β1 subunit knockout mice, which leads to membrane depolarization, vasoconstriction and systemic hypertension (Brenner, Perez et al. 2000). These studies provide a link between altered β1 subunit function, vascular reactivity and blood pressure. Whether SNPs in β1 subunits interfere with protein surface trafficking is unclear but would be relevant to investigate given findings reported here.

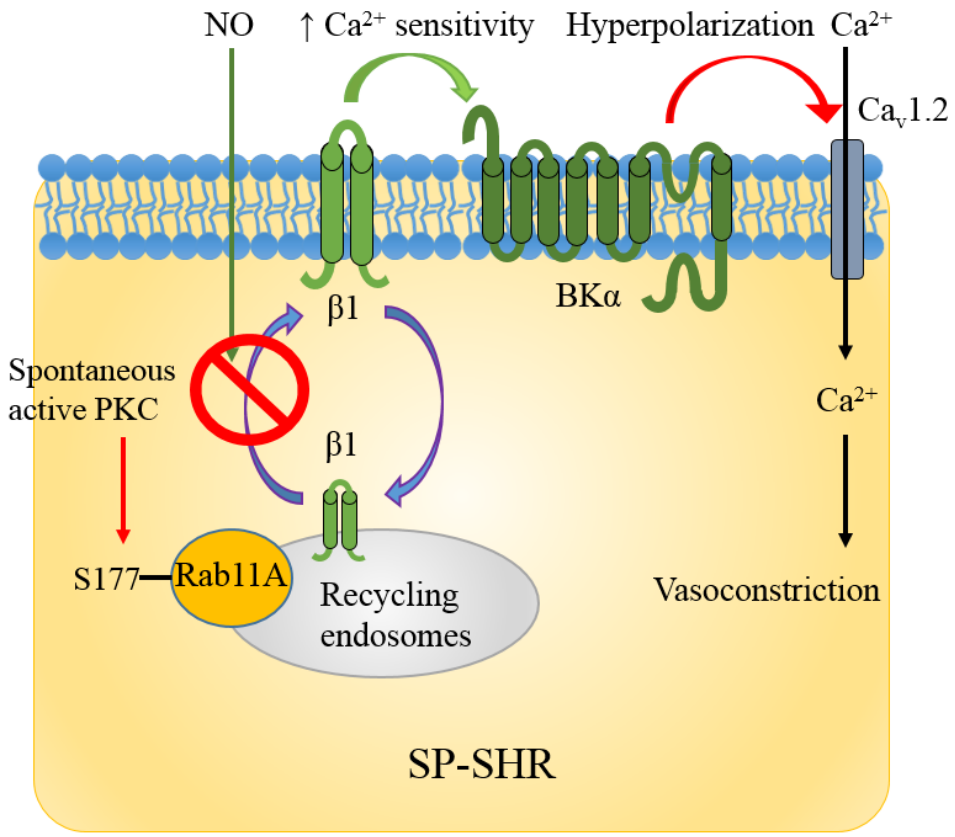


**Figure 5-2. Schematic figure shows the β1 subunit trafficking in arterial myocytes of SP-SHRs.**

*Notes:* Spontaneous active PKC blocks the stimulated surface trafficking of β1 subunits through Rab11A S177, which prevents BK channel activation in arterial myocytes of SP-SHRs.

The stimulated surface-trafficking of BK channel β1 subunits by either nitric oxide or membrane depolarization is inhibited in cerebral artery smooth muscle cells of stroke-prone spontaneously hypertensive rats (SP-SHR). Spontaneously active protein kinase C inhibits β1 subunit trafficking, which attenuates BK channel activation in arterial smooth muscle cells of SP-SHRs. PKC inhibition or overexpression of a phosphorylation-incapable Rab11A mutant (Rab11AS177A) restores stimulated β1 subunit trafficking and BK channel activation. PKC inhibition restores vasodilation to NO by enabling β1 subunit trafficking in cerebral arteries of SP-SHRs.

Hypertension is associated with vasoconstriction, attenuated NO-mediated vasodilation, and alterations in BK channel functionality, although interplay between these pathological modifications and mechanisms involved are unclear. The regulated surface-trafficking of BK channel α and β1 subunits in smooth muscle cells is a mechanism recently described to control arterial contractility. Whether BK channel subunit trafficking is altered during cardiovascular diseases and leads to changes in functionality is unclear.

Our data indicate that spontaneously active PKC inhibits β1 subunit trafficking in smooth muscle cells and is responsible for dysfunctional NO-induced BK channel activation and vasodilation in cerebral arteries of SP-SHRs.

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